(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081738 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/EP02/03891

(22) International Filing Date: 8 April 2002 (08.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/282,169 9 April 2001 (09.04.2001) US

(71) Applicant *(for all designated States except US)*: **FRAUNHOFER-GESELLSCHAFT ZUR FOERDERUNG DER ANGEWANDTEN FORSCHUNG E.V.** [DE/DE]; Leonrodstrasse 54, 80636 München (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BREDEHORST, Reinhard** [DE/DE]; Eichenstrasse 55, 20255 Hamburg (DE). **HINTSCHE, Rainer** [DE/DE]; Gravensteinstrasse 61C, 13127 Berlin (DE). **HEUBERGER, Anton** [DE/DE]; Auf dem Kyberg 21, 82041 Deisenhofen (DE).

(74) Agents: **OLGEMOELLER, Luitgard** et al.; Leonhard Olgemoeller Fricke, Postfach 10 09 62, 80083 München (DE).

(81) Designated States *(national)*: JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/081738 A2

(54) Title: SYSTEM AND METHOD FOR DETECTING AND QUANTIFYING NUCLEIC ACIDS USING COVALENT BINDING

(57) Abstract: Target nucleic acids bound to immobilized capture molecules by molecular biological or immunochemical reactions are detected with reactive residue-containing detector oligonucleotides and quantified with detector reagents capable of forming covalent linkages with the reactive residues on detector oligonucleotides. Alternatively, amplicons of target nucleic acids generated with reactive residue-containing primer oligonucleotides are bound to immobilized capture molecules by molecular biological or immunochemical reactions and quantified with detector reagents capable of forming covalent linkages with the reactive residues of captured amplicons. The quantity of reporter molecules provided or generated by covalently coupled detector reagents is a proportional measure of the quantity of target nucleic acids in the specimen. Enhanced covalent amplification systems include additional carrier systems for covalent attachment of multiple detector reagents and/or preformed covalently linked complexes of detector reagents.

SYSTEM AND METHOD FOR DETECTING AND QUANTIFYING NUCLEIC ACIDS USING COVALENT BINDING

I. FIELD OF THE INVENTION

The present invention relates to systems and assay procedures for the detection and quantification of nucleic acids and amplicons thereof using probes for or amplicons of the said nucleic acids, carrying reactive residues which may directly or indirectly be covalently linked to detector reagents.

II. BACKGROUND OF THE INVENTION

At present, the vast majority of assays suitable for sensitive detection of nucleic acids is based on enzyme-linked methods in combination with optical detection systems. Reporter systems utilized in these assays include detection by fluorescence, chemiluminescence, and colorimetry. The labels are generated via nucleic acid-enzyme conjugates similar to ELISA techniques.

While enzyme-linked assays for detecing and quantifying nucleic acids have gained wide acceptance in fields such as biotechnology, environmental protection, and public health, they pose several problems. The enzyme-linked detection step requires efficient binding of enzyme molecules to captured target nucleic acids. This is mediated via the interaction of affinity components. Most frequently, enzyme molecules are conjugated to streptavidin or hapten-specific antibodies. Such conjugates are bound to biotin or hapten residues attached directly to target nucleic acids or to nucleic acid reactants. In other enzyme-linked assay procedures, the enzyme molecules are derivatized with biotin residues and then complexed with streptavidin. Since each streptavidin molecule provides four biotin binding sites, these complexes are still capable of binding to biotinylated nucleic acids. In order to guarentee specificity and sensitivity of the detection procedure, it is mandatory that nonspecific binding of the affinity components to nucleic acids is negligible and that the binding affinity of the affinity components is sufficiently high to avoid dissociation and, thereby, loss of bound enzyme molecules during washing steps.

The biotin interaction with streptavidin is among the strongest non-covalent affinities known, exhibiting an affinity constant in the range of 10^{-15} M. However, streptavidin is expensive. As a result, assay procedures employing streptavidin are less cost effective. Unfortunately, for the detection of nucleic acids streptavidin cannot be substituted by the far less expensive avidin although both streptavidin and avidin bind biotin with similar affinity. Avidin has a strong tendency to bind nonspecifically to components other than biotin due to its high pI of about 10 and its carbohydrate content. The positive charge on the protein causes ionic interactions with the negatively charged nucleic acids. In contrast, streptavidin exhibits a much lower pI of 5-6 and is not glycosylated. These factors lead to better signal-to-noise ratios in assays using streptavidin-biotin interactions than those employing avidin-biotin. It is important to note, however, that in comparison to avidin nonspecific binding of streptavidin to nucleic acids is substantially reduced but still not eliminated.

Application of hapten-specific antibodies as affinity components in enzyme-linked nucleic acid assays pose similar problems. Many anti-hapten antibodies are less expensive than streptavidin, but provide much lower binding affinities than the streptavidin-biotin system. As a result, partial dissociation of bound antibody-enzyme conjugates during incubation and washing steps may occur. Only a few anti-hapten antibodies have been shown to bind the corresponding hapten with high affinity. Since the generation of high affinity anti-hapten antibodies is time-consuming and costly, assay procedures employing these antibodies are also less cost effective. Anti-fluorescein antibodies have displayed an unusual high affinity which makes fluorescein an ideal hapten for many detection schemes, but for nucleic acid detection procedures, fluorescein and other haptens are far away of being ideal. Some of these haptens are relatively bulky which leads to potential steric hindrance problems during the hybridization process. Others are very hydrophobic, thereby causing non-specific adsorption problems. Similarly, use of digoxigenin as a hapten only reduces but not eliminates the problems mentioned above.

III. SUMMARY OF THE INVENTION

It is the problem of the present invention to provide a system and a method for the detection and quantification of nucleic acids which substantially improves the systems known in the prior art in that it provides a cost effective procedure for fast analyses and/or with a high level of detection sensitivity. The method and the system should be useful with or without amplification of the nucleic acids to be measured.

This problem is solved by providing methods and components for a covalent system in which nucleic acids to be detected are bound to immobilized capture molecules and then contacted with residue-containing detector oligonucleotides or in which nucleic acids which have been amplified using reactive residue-containing primer oligonucleotides are bound to immobilized capture molecules. In both systems or methods, the bound residue-containing detector oligonucleotides or residue-containing amplified nucleic acids are detected and optionally quantified using detector reagents capable of forming covalent linkages with the reactive residues of the detector oligonucleotides or the amplified nucleic acids. The quantity of reporter molecules provided or generated by covalently coupled detector reagents is a proportional measure of the quantity of target nucleic acids in the specimen.

The invention comprises a system that consists of a solid-support with immobilized capture molecules, primer oligonucleotides for polymerase mediated amplification of the nucleic acid to be detected containing reactive residues R1 or detector oligonucleotides containing such reactive residues R1, respectively, and detector reagents containing detector units and reactive residues R2 capable of forming covalent linkages with reactive residues R1 of nucleic acids which had been amplified using primer oligonucleotides, or with reactive residues R1 of detector oligonucleotides.

Further, this invention provides a method for the detection or quantification of a nucleic acid in a liquid sample, in which a solid support having immobilized thereon capture molecules capable of binding to a first part but not to a second part of the nucleic acid to be detected or quantified or amplicons thereof is provided. The capture molecules bound on this solid support are contacted with the liquid sample, either with or without a

previous amplification step in which primer oligonucleotides for polymerase-mediated amplification of the nucleic acid to be detected and each containing at least one first reactive residue R1 are used for amplification. At least in case no amplification using the said R1-containing primer oligonucleotides has been performed, detector oligonucleotides having a sequence capable of binding to the said second part of the nucleic acid to be detected or quantified and containing at least a first reactive residue R1 are added. Next, unbound reactive detector oligonucleotides are removed, if required, and a detector reagent comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive R1 residues of the said detector oligonucleotides or of the amplicons of the nucleic acid to be detected, respectively, is added. After removing unbound detector reagent, the presence of reporter molecules provided or generated by the said detector units is detected and, if desired, quantified.

For enhanced covalent detection systems, the invention suggests to use enhancement components, e.g. carrier molecules which are preferably organic polymers and contain multiple reactive residues R3 for covalent coupling to reactive residues R2 of multiple detector reagents and/or to reactive residues R1 nucleic acids polymerized using the said R1 containing primer nucleotides or of the said detector oligonucleotides. Alternatively or in addition, the enhanced detection systems may include complexes of detector reagents containing reactive residues R2 capable of covalent binding to primer oligonucleotides or detector oligonucleotides, or to carrier molecules.

Suitable carrier molecules include but are not limited to oligomeric or polymeric derivatives of nucleotides, saccharides, amino acids, vinyl alcohols, vinyl pyrrolidinones, acrylic acids, various urethanes, and phosphazenes. Carrier molecules may be covalently attached to detector oligonucleotides, to nucleic acids amplified using the primer oligonucleotides of this invention, or to detector reagents in which case they will preferably carry more than one of the said component. Alternatively, they may be utilized as non-attached carrier molecules containing two different types of reactive residues R3, one being capable of covalent binding to detector oligonucleotides or nucleic acids amplified using primer oligonucleotides of this invention, and the other

being capable of covalent binding to detector reagents. In one example, the detector reagents may be or comprise polymeric carrier molecules containing more than one detecting unit, e.g. multiple enzyme molecules immobilized thereon and optionally (see below) derivatized with reactive residues R2. In another embodiment, the primer oligonucleotides or the detector oligonucleotides contain an oligomeric or polymeric carrier molecule covalently attached thereto, the carrier molecule carrying more than one of the said first reactive residues R1. Each enhancement component can be used alone or in combination with other enhancement components.

The detector units of detector reagents include but are not limited to enzymes capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules (also designated as "reporter molecules"), and liposomes containing encapsulated such reporter molecules. The said detector units may be likewise used in complexes containing more than one detector reagent, e.g. in covalently linked complexes of detector reagents.

The present invention provides a covalent detection system that eliminates the use of expensive affinity components. The invention also comprises a broad variety of detector reagents and enhancement components, which allows an optimal design of assay procedures according to the requirements of the application field. As a result, the present invention simplifies the development of assay components and reduces costs without compromising detection sensitivity.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates examples of reactive residue pairs capable of forming covalent linkages, i.e. a hydrazone linkage by reacting a hydrazide group as a first reactive residue and an aldehyde group as a second reactive residue, or vice versa; a secondary amine bond by reacting an amine group as a first reactive residue with an aldehyde group as a second reactive residue, or vice versa, the resulting Schiff base further being reduced by e.g. $NaCNBH_3$; a disulfide bond by reacting a sulfhydryl group as a first ractive residue or a second

reactive, respectively, with a pyridyldisulfide derivate containing the second reactive residue, or the first reactive residue, respectively, yielding pyridone-2-thione as a side product; and a thioether bond by reacting a sulfhydryl group as a first reactive residue with a iodoacetyl derivative as a second reactive residue, or vice versa.

Figure 2 illustrates the reactivity of iodoacetyl residues towards functional groups of proteins containing lysine, histidine, cysteine or methionine.

Figure 3 shows a first assay configuration using reactive residue-containing detector oligonucleotides.
Figure legend: solid support or sensor surface (1); surface bound capture oligonucleotide (2); target DNA (3); region of DNA double helix (4); detector oligonucleotide (5); flexible spacer molecule (6); reactive residue R1 (7) capable of forming a covalent linkage with reactive residue R2; detector reagent (DR) (8); reactive residue R2 (13) capable of forming a covalent linkage with reactive residue R1. The detector reagent 8 may be coupled to a polymeric carrier not shown in the figure.

Figure 4 shows a second assay configuration using polymerase-mediated amplification of the nucleic acid to be detected, wherein primer oligonucleotides are used which contain a reactive residue and thus, the amplicons of the amplification step are provided with the said reactive residue.
Figure legend: solid support or sensor surface (1); surface bound capture oligonucleotide (2); region of DNA double helix (4); flexible spacer molecule (6); reactive residue R1 (7) capable of forming a covalent linkage with reactive residue R2; detector reagent (DR) (8); primer oligonucleotide (9) containing reactive residue R1; target nucleic acid strand (10); modified target nucleic acid strand (11) containing reactive residue R1 (7); reactive residue R2 (13) capable of forming a covalent linkage with reactive residue R1. The detector reagent 8 may be coupled to a polymeric carrier not shown in the figure.

Figure 5 shows a third assay configuration which makes use of separate carrier molecules to enhance the covalent amplification assay.
Figure legend: solid support or sensor surface (1); surface bound capture oligonucleotide (2); region of DNA double helix (4); flexible spacer molecule (6); reactive residue R1 (7) capable of forming a covalent linkage with reactive residue R2; detector reagent (DR) (8); modified target nucleic acid strand (11) containing reactive residue R1; polymeric carrier molecule (12) carrying reactive residues R3 (13) capable of forming covalent linkages with reactive residues R1 and R2; reactive residue R2 (14) capable of forming a covalent linkage with reactive residue R3. In addition, the detector reagent 8 may be coupled to a polymeric carrier not shown in the figure.

V. DETAILED DESCRIPTION OF THE INVENTION

In its broadest aspect, this invention provides a technology for detecting and quantifying target nucleic acids or their amplicons for analytical and clinical applications by a combination molecular biological and covalent chemical reaction procedures. Applications of this invention include but are not limited to the examination of biological materials including body fluids and tissues for specific binding sites of effector substances (e.g., pharmacological or toxic molecules), the presence of infectious microorganisms, malignancies, inherited genetic defects, pharmacogenomics, forensic medical evidence, and paternity / maternity identification.

The invention employs a solid-support with immobilized capture molecules, reactive residue (R1) -containing primer oligonucleotides (see e.g. Fig. 4) or reactive residue (R1) -containing detector oligonucleotides (see e.g. Fig. 3), and detector reagents containing reactive residues R2 capable of forming covalent linkages with the reactive residues of primer oligonucleotides or detector oligonucleotides. Capture molecules include but are not limited to single-chain nucleic acids (ribo and deoxyribo nucleic acids), single-chain oligonucleotides (ribo and deoxyribo oligonucleotides), and 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues. 'Preorganized' oligonucleotide structures offer a potential advantage since

they exhibit a higher affinity for target nucleic acids provided they are rigidified prior to binding in a position that resembles the bound conformation.

Preferred detector reagents include or consist of enzymes containing or being derivatized with suitable reactive residues R2, if required, polymeric carrier molecules containing multiple immobilized enzyme molecules containing or being derivatized with suitable reactive residues R2, if required, or liposomes containing encapsulated reporter molecules and surface-attached reactive residues R2. Preferred examples of polymeric carrier molecules serving as enzyme carriers include oligomeric or polymeric derivatives of saccharides, amino acids, vinyl alcohols, vinyl pyrrolidinones, acrylic acids, various urethanes, and phosphazenes. In a more preferred embodiment, derivatives of dextran are employed as enzyme carriers. Preferred reporter molecules for encapsulation in liposomal detector reagents include a) chromogenic, fluorescent, and electrochemically detectable molecules, b) essential components of chemical detection systems such as non-enzymatic chemiluminescent reactions, c) essential, non-enzymatic components of enzymatic detection systems, d) enzyme activators, e) enzyme inhibitors, and f) enzymes capable of generating colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically active molecules. The release of reporter molecules from covalently coupled liposomes is effected by methods known in the art, e.g. by an increase of the ambient temperature or by the addition of liposome-lysing solvents such as organic solvents or detergents.

Reactive residues suitable for this invention include those which are sufficiently stable in aqueous media such as primary amines, hydrazides, aldehydes, sulfhydryls, pyridyl disulfides, TNB disulfides, sulfenylthiosulfate residues, vinylsulfone residues, certain maleimides, and halo acetyl residues. Preferred examples of reactive residue pairs capable of forming covalent linkages are pairs such as aldehydes and hydrazide residues, aldehydes and primary amines, pyridyl disulfide residues and sulfhydryl groups, vinylsulfone residues and sulfhydryl groups, maleimides and sulfhydryl groups, and halo acetyl residues and sulfhydryl groups (see e.g. Fig. 1). Hydrazone linkages formed by reaction of aldehydes with hydrazide compounds as well as Schiff bases formed by reaction of aldehydes with primary amines may be further stabilized by

reduction with compounds such as borohydride and cyanoborohydride. Suitable for this invention are also protected reactive residues such as thioester groups which can be cleaved by treatment with hydroxylamine, thereby generating free sulfhydryl groups.

Preferred reactive residues R1 of the said primer oligonucleotides and of the said detector oligonucleotides include those which minimize unwanted reaction with functional groups of proteins possibly present in the reaction mixture (e.g. via their amino groups), such as amines (preferably primary amines), hydrazides, sulfhydryls, pyridyl disulfides, TNB (2-nitrobenzoic acid) disulfides, sulfenylthiosulfate residues, vinylsulfone residues. Suitable for this invention are also protected reactive groups such as thioester residues which can be cleaved by treatment with hydroxylamine, thereby generating free sulfhydryl groups. However, since detector oligonucleotides may be applied after complete removal of proteins, if appropriate, they may also contain reactive residues such as aldehydes, ketone residues, maleimides, aryl halides, alkyl halides, and halo acetyl groups which are reactive with proteins.

The reactive residues R2 of the detector reagents may be selected from the same group of reactive residues as defined for R1 above, if the detector reagents are or comprise liposomes having surface-attached reactive residues R2 and containing encapsulated reporter molecules. Since liposomes can be employed in the absence of proteins, those reactive residues R1 mentioned above as useful in the absence of proteins are also suitable as surface-attached residues R2 of the said liposomes.

If the detector reagents are or include enzymes, they may optionally be derivatized with reactive residues R2. The choice of reactive residues R2 for derivatization of enzyme molecules depends on their intended application as detector reagents. If enzymes are employed as non-complexed detector reagents, protected reactive residues such as thioester groups, pyridyl disulfides, and 2-nitrobenzoic acid disulfides are required for derivatization to avoid unwanted complex formation. For the formation of enzyme complexes, a broad variety of reactive residues is suitable including those which are reactive with non-derivatized proteins. For a more controlled synthesis of enzyme complexes, however, enzyme molecules may be derivatized with a broad variety of

amine-reactive heterobifunctional cross-linking reagents prior to complex formation. In a specific embodiment, one part of enzyme molecules may be derivatized with other reactive residues than another part thereof, e.g. one part with pyridyl disulfide residues by reaction with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and the other part with free sulfhydryl groups by reaction with 2-iminothiolane (see e.g. Fig.1, third line). Using appropriate derivatization conditions, each enzyme molecule will be derivatized with a few reactive residues. Upon mixing of these two enzyme preparations, complexes will form via thiol-disulfide exchange. If disulfide bonding is found unsuitable for producing stable complexes, other cross-linking reagents may be used that form more stable bonds. For example, the heterobifunctional cross-linker N-γ-maleimidobutyryloxy succinimide (GMBS) reacts with primary amines, thus introducing a maleimide group. This maleimide group can subsequently react with a free sulfhydryl group, thereby forming a stable thioether linkage. If steric hindrance problems have to be solved, cross-linkers such as a long-chain version of SPDP can be used which introduce long spacer arms between the cross-linked enzyme molecules.

Non-derivatized enzyme molecules can also be employed as detector reagents provided the reactive residues R1 attached to detector oligonucleotides or primer oligonucleotides or carrier molecules are reactive with functional groups (reactive residues R2) of non-derivatized proteins. For example, iodoacetyl residues can react with a number of functional groups within proteins including the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and the primary ε-amino group of lysine residues and N-terminal α-amines (Fig. 2). The relative rate of reaction with each of these residues is dependent on the degree of ionization and thus the pH at which the modification is performed. The exception to this is the methioninyl thioether that reacts rapidly at nearly all pH values above about 1.7. The relative reactivity of iodoacetyl residues toward protein functionalities is sulfhydryl > imidazolyl > thioether > amine. Utilizing these reactivities of iodoacetyl groups, assays can be designed which require a minimum of chemical derivatization of assay components.

The components of the detection or quantification system of the present invention allow for the design of various assay configurations for the detection or quantification of a nucleic acid in a liquid sample.

A first basic procedure of the invention includes hybridization of target nucleic acids to immobilized capture oligonucleotides, detection of captured target nucleic acids by detector oligonucleotides via hybridization to single-stranded segments of captured target nucleic acids, covalent binding of detector reagents to the reactive residues of hybridized detector oligonucleotides, and quantification of reporter molecules provided or generated by covalently coupled detector reagents (Fig. 3). The quantity of reporter molecules provided or generated by covalently coupled detector reagents is a proportional measure of the amount of target nucleic acids in the sample. Thus, the first basic procedure comprises the following steps: (a) providing a solid support having immobilized thereon capture molecules capable of binding to a first part but not to a second part of the nucleic acid to be detected or quantificated or amplicons thereof, (b) contacting said capture molecules with the liquid sample possibly containing the nucleic acid to be detected, (c) adding detector oligonucleotides having a sequence capable of binding to the said second part of the nucleic acid to be detected or quantified and containing at least one of a first reactive residue R1, (d) removing unbound reactive detector oligonucleotides, (e) adding a detector reagent, comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive R1 residues of the said detector oligonucleotides or to third reactive residues R3 of molecules capable of being bound to the said first reactive residue R1 of the said detector oligonucleotides via a covalent linkage, (f) removing unbound detector reagent, and (g) detecting or quantifying the presence of reporter molecules provided or generated by the said detector units.

In a second basic procedure of the present invention, a method for the detection or quantification of a nucleic acid in a liquid sample is provided in which target nucleic acids are amplified using a suitable polymerase and primer oligonucleotides containing reactive residues. After hybridization of the resulting amplicons to immobilized capture oligonucleotides, detector reagents are covalently coupled to the reactive residues of

captured amplicons (Fig. 4). Further steps are identical with those of the first basic procedure. Thus, the second basic procedure provides a method comprising the following steps: (a) adding primer oligonucleotides for polymerase-mediated amplification of the nucleic acid to be detected and each containing at least one of a first reactive residue R1 to the said sample under conditions which allow amplification of the said nucleic acid, resulting in nucleic acid amplicons carrying the said first reactive residue R1, (b) providing a solid support having immobilized thereon capture molecules capable of binding to the nucleic acid to be detected or to amplicons thereof, (c) contacting said capture molecules with the liquid sample possibly containing nucleic acid amplicons amplified according to (a), (d) adding a detector reagent, comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the first reactive residue R1 of nucleic acid molecules amplified according to (a) or to third reactive residues R3 of molecules capable of being bound to the first reactive residue R1 of the said nucleic acid molecules amplified according to (a) via a covalent linkage, (e) removing unbound detector reagent, and (f) detecting or quantifying the presence of reporter molecules provided or generated by the said detector units.

Assay procedures of the present invention utilizing one or more enhanced components are specific embodiments based on the basic concepts.

In one preferred embodiment of enhanced covalent amplification systems, detector oligonucleotides containing multiple reactive residues R1 are utilized in the procedures as defined above, and preferably in the method defined above as first basic procedure. Preferred methods for derivatizing detector oligonucleotides with multiple reactive residues include covalent modification of the oligonucleotide termini with additional nucleotides containing reactive residues R1 or with hydrophilic carrier molecules containing multiple reactive residues R1. Preferred reactive residues for this application include those which are sufficiently stable in aqueous media such as primary amines, hydrazides, aldehydes, sulfhydryls, pyridyl disulfides, vinylsulfone residues, certain maleimides, and halo acetyl residues. Suitable carrier molecules include but are not

limited to oligomeric or polymeric derivatives of saccharides, amino acids, and vinyl alcohols.

In another preferred embodiment of the present invention using enhancing components, primer oligonucleotides containing at their 5'-termini multiple reactive residues R1 are utilized in combination with the second basic procedure. Preferred derivatized primer oligonucleotides are those which have been derivatized at their 5'-termini with multiple reactive residues, the modifications being identical with those described above for derivatizing detector oligonucleotides with multiple reactive residues R1. Preferred reactive residues for this application are those which minimize unwanted coupling of primer oligonucleotides to proteins present in the reaction mixture (e.g., via their amino groups). Such reactive residues include amines, hydrazides, pyridyl disulfides, vinylsulfone residues, and thioester residues.

In another preferred embodiment of the present invention using enhancing components, additional non-attached carrier molecules are utilized which contain reactive residues R3 being capable of covalent binding to reactive residues R1 of primer oligonucleotides or detector oligonucleotides as well as of covalent binding to reactive residues R2 of detector reagents. The reactive residues R3 of the said carrier molecules capable of covalent binding to primer oligonucleotides or detector oligonucleotides R1 and those being capable of covalent binding reactive residues of detector reagents R2 may be identical or different. Assays utilizing additional non-attached carrier systems (see Fig. 5) include covalent binding of carrier molecules to the reactive residues R1 of hybridized detector oligonucleotides, or to the reactive residues R1 of captured amplicons of target nucleic acids, respectively, wherein the covalently coupled carrier molecules are also able to covalently binding of reactive residues R2 of more than one detector reagent. Subsequently, the reporter molecules provided or generated by covalently coupled detector reagents are detected. Suitable carrier molecules include but are not limited to oligomeric or polymeric derivatives of saccharides, nucleotides, amino acids, vinyl alcohols, vinyl pyrrolidinones, acrylic acids, various urethanes, and phosphazenes. Copolymer preparations of two monomers are also applicable for this invention. Preferred reactive residues R3 for carrier molecules

include those which are sufficiently stable in aqueous media such as primary amines, hydrazides, aldehydes, sulfhydryls, pyridyl disulfides, vinylsulfone residues, certain maleimides, and halo acetyl residues.

In still another preferred embodiment of the present invention using enhancing components, covalently linked complexes of detector reagents are used. Each molecule of these complexes contains at least one detector unit, at least one second reactive residue R2 capable of forming a covalent linkage to the first reactive residues R1 of the amplicons amplified by said R1-containing primer oligonucleotides, or of the detector oligonucleotides present, respectively, or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotide or detector oligonucleotide via a covalent linkage, e.g. a carrier molecule containing more than one reactive residue R3 as defined above, and at least one fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2 of another of the complexed molecules, wherein reactive residues R2 and R4 of different molecules are covalently attached to each other.

In still another preferred embodiment, covalently linked complexes of detector reagents may be formed by chemical reaction of two types of detector reagents containing different types of reactive residues. The reactive residues R2 of type I detector reagents are capable of covalent binding to amplicons of target nucleic acids or to detector oligonucleotides, whereas the reactive residues R4 of type II detector reagents are capable of forming covalent linkages with those of type I detector reagents. Preferred examples of reactive residue pairs capable of forming covalent linkages include but are not limited to pairs such as aldehydes and hydrazide residues, aldehydes and primary amines, pyridyl disulfide residues and sulfhydryl groups, vinylsulfone residues and sulfhydryl groups, maleimides and sulfhydryl groups, and halo acetyl residues and sulfhydryl groups. Alternatively, covalently linked complexes of detector reagents may be formed by chemical reaction of one type of detector reagent using homobifunctional cross-linking reagents or polymers containing reactive residues capable of forming covalent linkages with those of the detector reagent.

Preferred cross-linked detector reagents include covalently linked complexes of derivatized enzyme molecules and/or of liposomes containing encapsulated reporter molecules and surface-attached reactive residues. Cross-linked detector reagents may be employed as preformed complexes in enhanced covalent amplification assays. Preferred methods for the preparation of preformed complexes of detector reagents include coupling procedures with and without additional spacer molecules. Especially preformed enzymes complexes may require additional spacer molecules to retain enzymatic activity. Useful spacer molecules include oligoethylene derivatives and the broad variety of homo- and heterobifunctional cross-linking reagents.

In still another preferred embodiment comprising an amplification component, the detector reagents contain polymeric carrier molecules to each of which more than one detector unit is attached. The detector units are preferably selected from enzymes immobilized to the polymeric carrier molecules and capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules.

The present invention provides systems and methods for the detection or quantification of nucleic acids in liquid samples that eliminate the necessity of expensive high affinity components. Covalent coupling steps avoid the potential loss of bound affinity components as a result of dissociation processes during incubation and washing steps. The invention also comprises a broad variety of detector reagents and enhancement components which allows an optimal design of assay procedures according to the requirements of the application field. For example, preformed complexes of detector reagents such as enzymes or reporter molecule-containing liposomes can be easily generated by mixing two preparations of the same detector reagent each derivatized with different reactive residues capable of forming covalent linkages with each other. Using such complexes of detector reagents, highly sensitive assays can be designed. Utilizing the reactivities of iodoacetyl groups towards several functional groups within proteins, the invention allows also to employ non-derivatized enzyme molecules as

detector reagents. As a result, the present invention simplifies the development of assay components and reduces costs without compromising detection sensitivity.

VI. DETAILED DESCRIPTION OF COMPONENTS OF THE INVENTION

VI.1. REACTIVE RESIDUES

As mentioned above, suitable reactive residues (R1 to R4) for this invention include primary amines, hydrazides, aldehydes, ketones, sulfhydryls, pyridyl disulfides, vinylsulfone residues, maleimides, aryl halides, alkyl halides, and halo acetyl residues. More preferred are those reactive residues which are sufficiently stable in aqueous media at neutral pH such as primary amines, hydrazides, aldehydes, sulfhydryls, pyridyl disulfides, vinylsulfone residues, and halo acetyl residues. Suitable for this invention are also protected reactive residues such as thioester groups which can be cleaved by treatment with neutral hydroxylamine, thereby generating free sulfhydryl groups. The system and the procedure of the present invention involve the reaction of two of these reactive residues to form covalent linkages. Preferred examples of reactive residue pairs capable of forming covalent linkages include pairs such as aldehydes and hydrazide residues, aldehydes and primary amines, pyridyl disulfide residues and sulfhydryl groups, vinylsulfone residues and sulfhydryl groups, maleimides and sulfhydryl groups, and halo acetyl residues and sulfhydryl groups.

VI.1.1. Chemical properties of preferred reactive residues

VI.1.1.1. Carbonyl residues

Carbonyl groups such as in aldehydes and ketones can be utilized as reactive residues for reaction with amine-containing compounds. Carbonyl groups react with amines forming Schiff base intermediates that are in equilibrium with their free forms. The interaction is pH-dependent, being especially efficient at high pH conditions. In a more preferred embodiment, the rather labile Schiff base interaction is chemically stabilized by reduction. The addition of sodium borohydride or sodium cyanoborohydride will result in reduction of the Schiff base intermediate creating a stable secondary amine linkage.

Aldehydes and ketones can further be utilized for reaction with derivatives of hydrazine, especially with hydrazide compounds formed from carboxylate groups. Reaction of hydrazides with either group creates hydrazone linkages. This bond is relatively stable if it is formed with a ketone, but somewhat labile if the reaction is formed with an aldehyde group. However, the linkage of a hydrazide with an aldehyde is much more stable than the easily reversible Schiff base interaction of an amine with an aldehyde. If further stabilization of the hydrazone linkage is required, sodium cyanpborohydride can be used to reduce the double bond and form a secure covalent linkage.

Further, dicarbonyl compounds such as glyoxal or phenylglyoxal derivatives may be utilized as reactive residues. The reaction of dicarbonyl compounds with a guanidinyl group such as that of an arginine residue creates a rather stable linkage due to the formation of a cyclic derivative.

VI.1.1.2. Disulfide residues

Pyridyl disulfide residues react readily with free sulfhydryls via disulfide exchange to yield single mixed disulfide conjugates. This is due to the fact that the pyridyl disulfide functional group contains a leaving group that is easily transformed into the nonreactive, electron-stabilized compound pyridine-2-thione. Since this leaving group does not possess a free thiol, it is not capable of participating in further mixed disulfide formation. Further, sulfhydryl groups activated with the leaving group 5-thio-2-nitrobenzoic acid (TNB) may be utilized as reactive residues. TNB-activated sulfhydryl groups react efficiently at physiological to slightly alkaline pH conditions with free thiols via disulfide exchange similar to pyridyl disulfides.

Alternatively, sulfhydryl groups activated with sodium tetrathionate may be utilized as reactive residues. The tetrathionate anion reacts with a sulfhydryl to create a somewhat stable active intermediate, a sulfenylthiosulfate. Upon coupling of another sulfhydryl-containing compound to this reactive residue, the thiosulfate leaving group is removed and a disulfide linkage formed.

VI.1.1.3. Maleimide residues

In another embodiment of this invention, maleimide residues are utilized as reactive residues. Maleimides (maleic acid imides) are derivatives of the reaction of maleic anhydride and ammonia. The double bond of this functional group can undergo an alkylation reaction with sulfhydryl groups to form stable thioether bonds. Maleimide reactions are specific for sulfhydryl groups in the pH range of 6.5 -7.5. At pH 7, the reaction of maleimides with sulfhydryls proceeds at a rate 1000-fold faster than with amines. At higher pH values maleimides exhibit also some reactivity with amino groups. The maleimide group also may undergo hydrolysis to an open maleimic acid form that is unreactive toward sulfhydryls. This ring-opening reaction typically happens faster the higher the pH becomes. Hydrolysis is also dependent on the type of chemical group next to the maleimide function. For example, the cyclohexane ring of 4-(N-maleimidimethyl)-cyclohexane-1-carboxylate provides increased stability to maleimide hydrolysis probably due to its steric effects and its lack of aromatic character. Accordingly, the adjacent phenyl ring of m-maleimidobenzoic acid allows much greater rates of hydrolysis to occur at the maleimide ring. Therefore, it is preferred to utilize those maleimide derivatives which provide sufficient stability to hydrolysis. Furthermore, such maleimide derivatives are employed only at slightly acid pH conditions to minimize ring-opening hydrolysis reactions.

VI.1.1.4. Haloacetyl residues

Haloacetyl residues such as iodoacetyl and bromoacetyl residues can react with a number of functional groups including sulfhydryls and primary amines. However, iodoacetyl and bromoacetyl residues have also been shown to react with both imidazolyl side chain nitrogens of histidine and the thioether of methionine. The relative rate of reaction with each of these residues is dependent on the degree of ionization and thus the pH at which the modification is performed. The exception to this is the methioninyl thioether that reacts rapidly at nearly all pH values above about 1.7. At pH values > 9 iodoacetyl residues may be utilized for reactions with amine-containing compounds, whereas reactions with primary amines such as ε-amino groups of proteins can be avoided at pH values < 7. The reaction of iodoacetyl residues with sulfhydryls is relatively fast at neutral pH leading to stable thioether bonds. The relative reactivity of iodoacetyl residues toward the different functionalities is sulfhydryl > imidazolyl >

thioether > amine. Among halo derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive.

VI.1.1.5. Benzyl halide residues

It is also possible to use benzyl halide residues as reactive residues. Aryl halide compounds such as fluorobenzene derivatives can be used to form covalent bonds with amine-containing molecules like proteins. The reaction of fluorobenzene-type compounds with amines involves nucleophilic replacement of the fluorine atom with the amine derivative creating a substituted aryl amine bond. Other nucleophilics such as thiol, imidazolyl, and phenolate groups can also react. The relative rate of reactivity for aryl compounds is F > Cl ~ Br > sulfonate.

VI.1.1.6. Alkyl halide residues

Alkyl halide residues such as bromoalkyl residues can react with sulfhydryls and primary amines in a similar fashion as do haloacetyl residues. The reaction involves nucleophilic replacement of the halogen with the sulfhydryl or amine derivative creating a thioether or a substituted alkyl amine bond, respectively.

VI.1.1.7. Thioester residues

Further, thioester residues may be utilized as reactive residues. Compounds derivatized with thioester residues contain protected sulfhydryls that can be stored without degradation and subsequently deprotected as needed with an excess of hydroxylamine. Since the protecting group can be removed without adding disulfide reducing agents such as dithiothreitol, thioester residues provide an important advantage if the presence of reducing agents affects the function of other assay components such as disulfide-containing proteins.

VI.1.2. Methods of introducing reactive residues

For many assay components it may be necessary to chemically modify the structure in order to provide specific reactive residues. This may be done by reacting an existing chemical group with a modification reagent that contains or produces the desired functional group upon coupling. For example, an amine may be converted to a

sulfhydryl or a carboxylate may be altered to yield an amine. This same type of modification strategy also can be used to create highly reactive residues from functional groups of rather low reactivity. For example, carbohydrate chains on glycoproteins may be modified with sodium periodate to transform rather unreactive hydroxyl groups into highly reactive aldehydes. Similarly, 5'-phosphate groups of DNA may be transformed to yield terminal amines or sulfhydryls.

VI.1.2.1. Introduction of sulfhydryl residues

Assay components containing disulfide residues which are not essential for their functional activity may be selectively reduced to form active sulfhydryls. For example, sulfhydryl residues are introduced by reacting amine-containing assay components with sulfhydryl-introducing reagents, e.g. 2-iminothiolane (Jue, R. et al., Biochemistry 17, 5399, 1978), N-acetyl homocysteine thiolactone (Benesch, R., and Benesch, R.E. Proc. Natl. Acad. Sci. USA 44, 848, 1958), and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Carlsson, J. et al., Biochem. J. 173, 723, 1978). SPDP-derivatized assay components are further treated with dithiothreitol or another reducing agent to generate free sulfhydryl residues.

VI.1.2.2. Introduction of pyridyl disulfide residues

In order to provide pyridyl disulfide residues, amine-containing assay components may be reacted with pyridyl disulfide-introducing reagents, for example with SPDP (Carlsson, J. et al., Biochem. J. 173, 723, 1978). Alternatively, assay components containing sulfhydryl residues may be reacted with 4,4'-dipyridyl disulfide (Grasseti, D.R., and Murray, J.F. Arch. Biochem. Biophys. 119, 41, 1967) or 2,2'-pyridyl disulfide (Brocklehurst, K. et al., Meth. Enzymol. 34, 531, 1974). Further, aldehyde-containing assay components may be reacted with carbonyl-reactive pyridyl disulfide-introducing reagents, e.g. 3-(2-pyridyldithio) propionyl hydrazide (PDPH) (Zara, J.J. et al., Anal. Biochem. 194, 156, 1991).

VI.1.2.3. Introduction of TNB disulfide residues

Sulfhydryl residues of assay components may be chemically modified with the leaving group 5-thio-2-nitrobenzoic acid (TNB) to provide TNB disulfide residues. This may be

done by reacting assay components containing sulfhydryl residues with Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman, G.L. Arch. Biochem. Biophys. 82, 70, 1959).

VI.1.2.4. Introduction of sulfenylthiosulfate residues

Sulfhydryl residues of assay components may chemically be modified with sodium tetrathionate to provide sulfenylthiosulfate residues (Pihl, A., and Lange, R. J. Biol. Chem. 237, 1356, 1962).

VI.1.2.5. Introduction of thioester residues

In order to provide thioester residues, amine-containing assay components may be reacted with thioester-introducing reagents, e.g. N-succinimidyl S-acetylthioacetate (SATA) (Duncan, R.J.S. et al., R. Anal. Biochem. 132, 68, 1983), N-succinimidyl S-acetylthiopropionate (SATP) (Fuji, N. et al., H. Chem. Pharm. Bull. 33, 362, 1985), and S-acetylmercaptosuccinic anhydride (SAMSA) (Klotz, I.M., and Heiney, R.E. Arch. Biochem. Biophys. 96, 505, 1962).

VI.1.2.6. Introduction of haloacetyl residues

In order to provide haloacetyl residues such as iodoacetyl or bromoacetyl residues, amine-containing assay components may be reacted with haloacetyl-introducing reagents, e.g. N-hydroxysuccinimidyl iodoacetate (Rector, E.S. et al., J. Immunol. Meth. 24, 321, 1978), N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB) (Weltman, J.K. et al., BioTechniques 1, 148, 1983), N-succinimidyl 6-[(iodoacetyl)-amino] hexanoate (SIAX) (Brinkley, M. Bioconjugate Chem. 3, 2, 1992), N-succinimidyl 4-[((iodoacetyl)-amino)methyl] cyclohexane-1-carboxylate (SIAC), and p-nitrophenyl iodoacetate (NPIA) (Hudson, E.N., and Weber, G. Biochemistry 12, 4154, 1973).

VI.1.2.7. Introduction of maleimide residues

In order to provide maleimide residues, amine-containing assay components may be reacted with maleimide-introducing reagents, e.g. succinimidyl 4-(N-maleimido methyl) cyclohexane-1-carboxylate (SMCC) (Hashida, S., and Ishikawa, E. Anal. Lett. 18(B9), 1143, 1985), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), and N-γ-

maleimidobutyryloxy) succinimide ester (GMBS) (Fujiwara, K. et al., J. Immunol. Meth. 112, 77, 1988). Further, aldehyde-containing assay components may be reacted with carbonyl-reactive maleimide-introducing reagents, e.g. 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH) (Chamow, S.M. et al., Bioconjugate Chem. 5, 133, 1994), and 4-(N-maleimido methyl) cyclohexane-1-carboxyl-hydrazide (M_2C_2H).

VI.1.2.8. Introduction of carbonyl residues

In order to provide carbonyl groups such as aldehydes, amine-containing assay components may be reacted with aldehyde-introducing reagents, e.g. succinimidyl p-formylbenzoate (SFB) (Ghosh, S.S. et al., Anal. Biochem. 178, 43, 1989), and succinimidyl p-formylphenoxyacetate (SFPA). Alternatively, carbohydrate- or adjacent diol-containing assay components may be oxidized by treatment with periodate to generate aldehyde functions. Periodate cleaves carbon-carbon bonds that possess adjacent hydroxyls, oxidizing the hydroxyl groups to highly reactive aldehydes. Periodate oxidation of assay components with terminal cis-glycols will result in the loss of one carbon atom as formaldehyde and the creation of an aldehyde group on former number two carbon atom. Varying the concentration of sodium periodate during the oxidation reaction provides some specificity with regard to what carbohydrate residues of the assay components are modified. For example, the concentration of sodium periodate during the oxidation reaction may be adjusted to 1 mM for specific cleavage of the hydroxyls between carbon atoms 7, 8, and 9 of sialic acid residues. The product is the formation of one aldehyde residue on the number seven carbon and release of two molecules of formaldehyde. Oxidation of carbohydrate-containing assay components using 10 mM or greater concentrations of sodium periodate will result in the cleavage of adjacent hydroxyl-containing carbon-carbon bonds on other carbohydrate residues besides just sialic acid residues.

VI.1.2.9. Introduction of hydrazide residues

In order to provide hydrazide residues, aldehyde- or ketone-containing assay components may be reacted with bis-hydrazide reagents, e.g. carbohydrazide or adipic acid dihydrazide. The resulting hydrazone linkage may be further stabilized by reduction with sodium borohydride or sodium cyanoborohydride. Bis-hydrazide

reagents may also be employed for modification of carboxylate- and alkylphosphate-containing assay components. Carboxylates are first activated with other compounds to make them reactive toward bis-hydrazide reagents. In organic solutions, this may be accomplished by using a water-insoluble carbodiimide or by creating an intermediate active ester such as an N-hydroxysuccinimide ester. In aqueous solutions, water-soluble carbodiimides may be employed. Alkylphosphate residues such as those present at the 5'-terminus of RNA and DNA molecules require also activation prior to reaction with bis-hydrazide reagents. For example, activation of 5'-terminal alkylphosphate residues may be accomplished by reaction with imidazole in the presence of a water-soluble carbodiimide. The resulting phosphorimidazolide derivative is reactive toward bis-hydrazide reagents, releasing imidazole and forming a phosphoramidate bond (Ghosh, S.S. et al., Anal. Biochem. 178, 43, 1989).

VI.1.2.10. Introduction of amine residues

In order to provide amine residues, sulfhydryl-containing assay components may be reacted with amine-introducing reagents, e.g. 2-bromethylamine, and N-(iodoethyl) trifluoroacetamide (Schwartz W.E. et al., Anal. Biochem. 106, 43, 1980). Using appropriate conditions, the haloalkyl group of N-(iodoethyl) trifluoroacetamide specifically reacts with sulfhydryls to form the aminoalkyl derivative in one step.

VI.2. CAPTURE OLIGONUCLEOTIDES

As mentioned before, the invention comprises a solid support with immobilized capture oligonucleotides for specific binding of target nucleic acids. Capture oligonucleotides may be selected e.g. from single-chain nucleic acids (ribo and deoxyribo nucleic acids) and single-chain oligonucleotides (ribo and deoxyribo oligonucleotides). The oligonucleotides are designed to preferably form specific helical complexes with target nucleic acids to be detected in a sample. In a more preferred embodiment, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues are used as capture oligonucleotides (for a review, see Kool, E.T. Chem. Rev. 97, 1473, 1997). The rational for using 'preorganized' oligonucleotide structures is based on the observation that the affinity of oligonucleotides for the target nucleic acid can be increased by modifications rigidifying the oligonucleotide prior to binding so that

it more resembles the bound conformation. An oligonucleotide rigidly held in the binding position prior to complexation shows less free internal bond rotations that need to be 'frozen' during complexation. At the same time, the molecule is organized into a shape which is more complementary to the desired target than to undesired ones. This increases selectivity because mismatched targets will cause unfavorable responses such as non-optimum bond angles or steric clashes.

Strategies for the construction of 'preorganized' oligonucleotide structures include i) enhancement of base stacking, ii) limitation of bond rotations, and iii) linking of binding domains. Double-, triple-, and quadruple-stranded nucleic acid helices are stabilized by base stacking and hydrogen bonding interactions. Since the majority of the base-stacking interaction in nucleic acids is between bases within a strand, the strengthening of stacking will have the tendency to cause single-stranded oligonucleotides to become preorganized into a more regular helical conformation. This will therefore favor complexation by lowering the entropic cost. Preferred strategies for increasing stacking include but are not limited to the addition of simple substituents to DNA bases (e.g., methylation of pyrimidines at C-5), an increase of the surface area of DNA bases (e.g., by addition of aromatic heterocyclic groups to the C-5 position of pyrimidines), and the use of nonpolar DNA base analogues.

Examples for strategies for limiting bond rotations prior to complexation employ covalent bonds and include the synthesis of i) backbones with restricted freedom, ii) bicyclo-DNA, iii) hexose-DNA, and iv) circular DNA. Preferred examples of nucleic acid derivatives with rigid backbones include peptide nucleic acid (PNA) analogues containing amide bonds. Since an amide has restricted rotation about the carbonyl-nitrogen bond, PNAs are capable of forming strong duplexes with DNA at lowered ionic strength and very strong triplexes even at normal ionic strength. In the bicyclo-DNA approach, the normally flexible furanose ring is rigidified by addition of an ethylene bridge from C-3' to C-5', thereby forming a second five-membered ring to the natural structure. Since five-membered rings are considerably more flexible than six-membered rings, DNA analogues have been synthesized in which the furanose ring is expanded to a six-membered ring. In some cases, such oligonucleotide analogues

hybridized more strongly to DNA than the natural furanose-based structures. Another preferred way to significantly limit the conformational freedom of a flexible oligonucleotide is to cyclize the chain. A circular oligonucleotide can bind a single-stranded target RNA or DNA by forming standard Watson-Crick bonds. However, such binding is limited because of the helical twist of DNA.

It is possible to use the linking of binding domains as a strategy for preorganization. One aspect of critical importance for this strategy is the design of the linking group or groups. For optimal preorganization and thus highest affinity and selectivity, a linker should be both rigid and orient the binding domains in the productive geometry. Noncovalent links between binding domains have the advantage of simplifying the synthesis, but have the disadvantage of being relatively weak, thereby limiting effective preorganization. Preferred are covalent links between binding domains. For example, thiols may be placed into opposite strands of a duplex-forming sequence and used for disulfide cross-linking upon oxidation. Such duplexes become stabilized thermodynamically, presumably because of the entropic benefit.

Using covalent links between domains, preorganized oligonucleotide structures can be prepared that lead to triplex formation on single-stranded targets. Triple-helical nucleic acid structures are known since 1957. A purine DNA base can form hydrogen-bonded contacts on two sides, one termed the Watson-Crick face and the other Hoogsteen face. Thus, in duplex DNA, a purine stretch presents sites in the major groove for Hoogsteen complexation by a third strand. Single-stranded DNA can also serve as a target for triple helix formation, since a purine stretch can be bound on two sides by a molecule carrying both a Watson-Crick complementary domain and a Hoogsteen complementary domain. A second motif for triplex formation is the so-called purine motif, in which purine-purine-pyrimidine base triads are formed. As with the previous motif, a purine strand is in the middle, with two other strands forming hydrogen-bonded contacts. In such a motif, the Watson-Crick complementary pyrimidine strand represents the target and the other two strands the binding domains which are preorganized by linking.

A simple way to link two such triplex-forming domains is to connect them with extra non-pairing nucleotides or by a non-nucleotide linker such as hexaethylene glycol (clamp or fold-back oligonucleotides). A preferred strategy is to link two triplex-forming domains by two loops at both ends using nucleotide loops or non-nucleotide linkers (circular and looped oligonucleotides). Clamp-like oligonucleotides have been shown to bind target sequences with an 11°C advantage in T_m, whereas closure of the clamp into a full circle gave a 19°C advantage.

The clamp and circular oligonucleotide approaches are strategies in which two DNA-binding domains are linked at their end or ends. Another preferred approach is to link them across the center, thereby generating molecules with an 'H'-form. Examination of the base triads involved in triple helix shows that a bridge can easily link two C-5 positions on pyrimidines in opposite strands. Experimental results have shown that such a molecule cross-linked by a disulfide bridge (via thiopropyne-substituted thymidine nucleosides) binds a target strand more strongly than a clamp-like oligonucleotide (Chaudhuri, N.C., and Kool, E.T. J. Am. Chem Soc. 117, 10434, 1995). In a more preferred embodiment, the same strategy is used to cross-link a circular oligonucleotide with a disulfide bridge across the center. Thermal denaturation studies have shown that such bicyclic oligonucleotides bind complementary DNA strands with extremely high affinity (see Chaudhuri et al., above). Sequence selectivity was also found to benefit from this additional preorganization strategy.

Alternatively, it is possible to link together multiple binding domains (tethered DNA). For maximum cooperativity, a rigid linking domain is preferred. Although flexible linkers may not maximize affinity and selectivity, they may provide utility in some cases. For example, flexible tethers may be used to link two DNA-binding sequences for hybridization to separate sites such as purine stretches separated by non-homopurine segments.

VI.3. IMMOBILIZATION OF CAPTURE OLIGONUCLEOTIDES

The solid support serving for immobilization of capture molecules (capture oligonucleotides) may be chosen without restriction. For example, it may have the form

of glassy or polymeric beads, microtiter plates, porous, impervious, fibrous, or metallic matrices or membranes or the like, as is well-known in the art. Such matrices may be utilized as derivatized (e.g., by adsorption of polylysine, phenylalanine-lysine, or octadecylamine) or non-derivatized solid supports for immobilization of capture molecules.

Immobilization of capture oligonucleotides or derivatives thereof may be accomplished non-covalently or covalently by any of the well-known chemical coupling methods. For most applications, covalent immobilization techniques are preferred. Electrically mediated coupling procedures are equally applicable for use with the current invention. Also applicable for this invention are non-covalent, non-adsorptive immobilization techniques. For example, capture molecules derivatized with biotin residues may be immobilized onto solid supports functionalized by adsorption or covalent binding of streptavidin beforehand.

VI.3.1. Immobilization of capture oligonucleotides

The methods by which capture oligonucleotides may be derivatized with reactive residues for immobilization onto solid supports are numerous. Useful methods are those which allow selective derivatization of the termini to guarantee efficient hybridization with target nucleic acids or amplicons thereof. Subsequently, examples are given.

VI.3.1.1. Chemical modification of the terminal 5'-phosphate group of capture oligonucleotides

Capture oligonucleotides containing a 5'-phosphate group may be derivatized with amine or sulfhydryl terminal spacer molecules for immobilization onto amine-reactive or sulfhydryl-reactive solid supports (Hermanson, G.T. Bioconjugate techniques, Academic Press, San Diego, 1996). For example, the 5'-phosphate groups of capture oligonucleotides may be reacted with carbodiimide in the presence of imidazole to form active phosphorimidazolide intermediates. These derivatives are highly reactive with diamines or bis-hydrazide compounds, forming amine terminal spacer molecules via phosphoramidate linkages. Derivatization of the 5'-phosphate groups with cystamine

creates an amine terminal spacer containing a disulfide group. Reduction of the cystamine-labeled oligonucleotide using a disulfide reducing agent releases 2-mercaptoethylamine and generates a terminal thiol group.

Alternatively, sulfhydryl groups may be introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using S-triphenylmethyl O-methoxymorpholinophosphite derivatives of 2-mercaptoethanol, 3-mercaptopropan (1) ol, or 6-mercaptohexan(1)ol (Connolly, B.A., and Rider, P., Nucleic Acids Res. 13, 4485, 1985). After clevage from the resin and removal of the phosphate and base protecting groups, oligonucleotides are obtained which contain an S-triphenylmethyl group attached to the 5'-phosphate group via a two, three, or six carbon chain. The triphenylmethyl group can be readily removed with silver nitrate to give the free thiol. In another embodiment, primary amino groups can be introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol (Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987). After cleavage from the resin and removal of the phosphate and base protecting groups, a monomethoxytrityl-$NH(CH_2)_3PO_4$ - oligomer is obtained. The monomethoxytrityl group can be removed with acetic acid to give the amine-containing oligonucleotide.

VI.3.1.2. Chemical attachment of nucleotide derivatives to the termini of capture oligonucleotides

Nucleotide derivatives suitable for immobilization onto solid supports may be incorporated into capture oligonucleotides during automated chemical oligonucleotide synthesis. For example, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) (Kuijpers, W.H.A. et al., Bioconjugate Chem. 4, 94, 1993). After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, the derivatized oligonucleotide can be immobilized onto amine-containing solid supports via reductive amination. Other nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine or protected sulfhydryl groups are equally applicable for this invention.

VI.3.1.3. Enzymatic attachment of nucleotide derivatives to the termini of capture oligonucleotides

In another embodiment, nucleotide derivatives containing reactive residues are incorporated into capture oligonucleotides by enzymatic means as known in the art. Using enzymatic techniques, it is important that the reactive residue is incorporated into the nucleoside triphosphate in a way that does not affect enzyme recognition and activity. Examples of purine nucleotides include dATP derivatized with a reactive residue at its N-6 position or C-8 position via long linker arms. For example, 8-aminohexyl-dATP is a preferred derivative for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). Preferred examples of pyrimidine nucleotides include but are not limited to dUTP and dCTP modified with a reactive residue at their C-5 position via long linker arms.

VI.3.2. Immobilization of fold-back or looped preorganized capture oligonucleotides

Preorganized fold-back or looped capture oligonucleotides may be modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine or protected sulfhydryl groups via long spacer arms. After deprotection, such derivatized preorganized fold-back or looped capture oligonucleotides can be immobilized onto amine- or sufhydryl-recative solid supports or further derivatized with heterobifunctional reagents. Alternatively, preorganized fold-back or looped capture oligonucleotides may be formed by non-nucleotide linker molecules providing a functional group for immobilization onto a solid support in addition to the other two functional groups required for the formation of a preorganized oligonucleotide structure by cross-linking of the termini. A convenient molecule from which to build trifunctional linker molecules is the amino acid L-lysine. Its three functional groups, α-carboxy, α-amino, and ε-amino, can be derivatized independently to contain three arms carrying different reactive groups.

VI.3.3. Introduction of spacer molecules between solid support and capture oligonucleotides

In order to provide flexibility to oligonucleotides attached to solid supports, spacer molecules may be incorporated between the solid support and the oligonucleotide termini. Preferred spacer molecules are sufficiently long and flexible to allow efficient hybridization of the immobilized oligonucleotides with captured target nucleic acids. Preferred examples of spacer molecules include but are not limited to derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl) amino] hexanoate), providing a spacer arm with 14 atoms, oligomeric derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl)amino]-hexanoate), and oligoethylene glycol derivatives (Levenson, C., and Chang, C. Nonisotopically labelled probes and primers. In: PCR Protocols: A Guide to Methods and Applications [M.A. Innis et al., eds.] pp. 99-112, Academic Press, San Diego, 1990). Utilization of oligoethylene glycol as spacer molecules offers several advantages since oligoethylene glycol provides water solubility and a great degree of freedom for the linked oligonucleotides. Preferred are heterobifunctional derivatives of oligoethylene glycol including but not limited to those providing an amine-reactive residue such as a succinimidyl ester on one end and a sulfhydryl-reactive residue such as a maleimide or vinylsulfone residue on the other end. Since vinylsulfone residues are hydrolytically stable, they represent especially useful reactive residues for a second coupling step. Useful are also derivatives of oligoethylene glycol containing a t-Boc-protected amine on one end and an unprotected amine or an amine-reactive residue on the other end. After reaction of the unprotected functional residue, the t-Boc protecting group can be easily removed by treatment with trifluoroacetic acid. Thus, a wide range of derivatives of oligoethylene glycol may be used as spacer molecules between the solid support and the oligonucleotide termini. Conjugation of capture oligonucleotides or derivatives thereof to spacer molecules may be accomplished by any of the well-known chemical coupling methods.

In one embodiment, primary amino groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol as mentioned above. Thereafter, the terminal amino group may be derivatized with an amine-reactive

heterobifunctional reagent containing a long spacer arm such as succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP), succinimidyl 6-[6-{((iodoacetyl) amino) hexanoyl}amino]hexanoate (SIAXX), or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. The sulfhydryl-reactive residue of such reagents may be used for coupling of the oligonucleotide-spacer conjugates to thiol-containing solid supports.

In another embodiment, nucleotide derivatives containing reactive residues for covalent attachment of spacer molecules are incorporated into oligonucleotides by enzymatic means, as known in the art. For example, 8-aminohexyl-dATP may be utilized for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). The terminal amino group of the incorporated nucleotide may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Using this approach, oligonucleotides can be derivatized at their 3'-terminus with a variety of spacer arms including 6-[6-((amino)hexanoyl)amino]-hexanoate and oligoethylene glycol derivatives of various length. The terminal sulfhydryl-reactive groups of such spacer molecules can be used for subsequent coupling of the oligonucleotide-spacer conjugates to sulfhydryl-containing solid supports. Alternatively, the pyridyl disulfide residues of LC-SPDP-derivatized oligonucleotides can be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide-spacer conjugates to sulfhydryl-reactive solid supports.

In another embodiment, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) (Kuijpers, W.H.A. et al., Bioconjugate Chem. 4, 94, 1993). After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, 6-[6-((amino)hexanoyl)amino]hexanoate or oligoethylene glycol derivatives containing an amine residue on one end and a carbyoxyl residue on the other end may be coupled to the aldehyde groups of the oligonucleotide via reductive amination. Thereafter, the terminal carboxyl residues of

the spacer molecules may be activated for subsequent coupling of the oligonucleotide derivative to amine-containing solid supports.

In still another embodiment, preorganized fold-back or looped oligonucleotides are modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine groups via long spacer arms (e.g., a protected derivative of 8-aminohexyl-dATP). After deprotection, such derivatized preorganized fold-back or looped oligonucleotides may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Alternatively, preorganized fold-back or looped oligonucleotides are formed by non-nucleotide linker molecules such as L-lysine providing a functional group for covalent attachment of spacer molecules in addition to the other two functional groups required for formation of the preorganized oligonucleotide structure. Preferred examples of trifunctional linker molecules include but are not limited to L-lysine residues in which the α-amino and ε–amino groups are derivatized with hydroxyl terminal spacer molecules and the α-carboxyl group with derivatives of oligoethylene glycol spacer molecules containing a t-Boc-protected amine on one end and an unprotected amine on the other end. The terminal t-Boc protecting group can be removed by treatment with trifluoroacetic acid.

VI.4. PRIMER AND DETECTOR OLIGONUCLEOTIDES

In one important embodiment of this invention, ribo and deoxyribo oligonucleotide derivatives containing reactive residues are employed as primer and detector oligonucleotides. The primer oligonucleotides are designed to amplify target nucleic acids by enzymatic means and, thereby, to modify the target nucleic acids with specific reactive residues. The detector oligonucleotides are designed to form specific helical complexes with single-stranded segments of captured target nucleic acids. In another embodiment, 'preorganized' oligonucleotide structures containing reactive residues are used as detector oligonucleotides. The rational for using preorganized' oligonucleotide structures as detector oligonucleotides and strategies for the construction of 'preorganized' oligonucleotide structures are described in detail in section VI.2.

VI.4.1. Derivatization of primer and detector oligonucleotides with reactive residues

Reactive residues suitable for derivatization of primer oligonucleotides include e.g. amines, hydrazides, pyridyl disulfides, TNB disulfides, sulfenylthiosulfate residues, vinylsulfone residues, and thioester residues. These residues minimize unwanted coupling of primer oligonucleotides to proteins present in the reaction mixture. Since detector oligonucleotides can be applied after complete removal of proteins, they may also contain protein-reactive residues such as aldehydes and haloacetyl groups.

The methods by which primer and detector oligonucleotides may be derivatized with reactive residues are numerous and well-known in the art. Preferred methods include those which allow selective derivatization of the termini to guarantee efficient hybridization of the oligonucleotides with target nucleic acids. Primer oligonucleotides are derivatized at their 5'-termini only to allow for efficient polymerase-mediated extension at their 3'-termini. The chemical and enzymatic procedures for derivatization of primer and detector oligonucleotides with reactive residues via modification of the terminal 5'-phosphate group or via introduction of additional terminal nucleotide derivatives are identical with those described in detail in section VI.3.1.

In order to minimize potential interference of covalently attached reactive residues with the hybridization process and to provide optimal accessibility of reactive residues on hybridized oligonucleotides for subsequent coupling steps, spacer molecules may be incorporated between the oligonucleotide termini and the reactive residues. Examples of sufficiently long and flexible spacer molecules are monomers or dimers of 6-aminohexanoic acid and oligoethylene glycol derivatives. The chemical and enzymatic procedures for derivatization of primer and detector oligonucleotides with reactive residues via spacer molecules are identical with those described in detail in section VI.3.3.

VI.4.2. Derivatization of primer and detector oligonucleotides with multiple reactive residues

In one preferred embodiment of enhanced covalent amplication systems, detector and primer oligonucleotides derivatized with multiple reactive residues are utilized. Methods for derivatizing detector oligonucleotides with multiple reactive residues are e.g. covalent modification of the oligonucleotide termini with multiple additional nucleotides carrying reactive residues or with hydrophilic carrier molecules containing multiple reactive residues. Methods for derivatizing primer oligonucleotides with multiple reactive residues are e.g. covalent modification of the oligonucleotide 5'-terminus with multiple additional nucleotides carrying reactive residues or with hydrophilic carrier molecules containing multiple reactive residues. Suitable hydrophilic carrier molecules include but are not limited to derivatives of synthetic and natural oligomers or polymers of saccharides, amino acids, and vinyl alcohols. Copolymer preparations of two monomers are also applicable for this invention.

Reactive residues suitable for derivatization of carrier molecules or nucleotide tails attached to primer oligonucleotides include amines, hydrazides, pyridyl disulfides, TNB disulfides, sulfenylthiosulfate residues, vinylsulfone residues, and thioester residues. As mentioned above, these residues minimize unwanted coupling of the primer oligonucleotide derivatives to proteins present in the reaction mixture. Reactive residues suitable for derivatization of carrier molecules or nucleotide tails attached to detector oligonucleotides include also protein-reactive residues such as aldehydes and haloacetyl groups since the detector oligonucleotide derivatives can be applied after complete removal of proteins.

In order to minimize potential interference of covalently attached carrier molecules or tails consisting of multiple nucleotide derivatives with the hybridization process, spacer molecules may be incorporated between the oligonucleotide termini and the carrier molecules or nucleotide tails. Examples of sufficiently long and flexible spacer molecules are monomers or dimers of 6-aminohexanoic acid and oligoethylene glycol derivatives. As described in detail in sectionVI.3.3., a large variety of oligoethylene

glycol derivatives is available that may be used as spacer molecules between the carrier molecules or nucleotide tails and the oligonucleotide termini.

VI.4.2.1. Utilization of polymeric dextran as carrier of multiple reactive residues

In one embodiment of this invention, derivatives of dextran polymers are employed as polymeric carrier of multiple reactive residues. Dextran is mainly a linear polysaccharide consisting of repeating units of D-glucose linked together in glycosidic bonds wherein the carbon-1 of one monomer is attached to the hydroxyl group at the carbon-6 of the next residue. Occasional branch points also may be present in a dextran polymer, occurring as α-1,2, α-1,3, or α-1,4 glycosidic linkages. The monomers contain at least three hydroxyls that may undergo derivatization reactions. This multivalent nature of dextran allows reactive residues to be attached at numerous sites along the polymer chain.

In one embodiment, dextran polymers are used which are oxidized with periodate to produce aldehydes. This procedures results in two aldehyde groups formed per glucose monomer, thus producing a highly reactive, multifunctional polymer able to couple with numerous amine-containing molecules (Bernstein, A. et al., J. Natl. Cancer Inst. 60, 379, 1978).

In another embodiment, polyaldehyde dextran is used which is further derivatized with amine-containing homobifunctional reagents such as ethylene diamine, diaminodipropylamin (3,3"-imino *bis*propylamine), or the short-chain Jeffamine derivative EDR-148 (Texaco Chem. Co.) containing a hydrophilic, polyether, 10-atom chain. These compounds are conjugated by Schiff base formation followed by reductive amination to create stable secondary (or tertiary amine) linkages. The optimal pH for the reductive amination reaction is an alkaline environment between pH 7 and 10.

Further, it is possible to use carboxyl-terminal dextran derivatives which are prepared by coupling amine-containing heterobifunctional reagents such as 6-aminocaproic acid or β-alanine to polyaldehyde dextran. Another embodiment uses reactive alkyl halogen

compounds containing a terminal carboxylate group on the other end such as chloroacetic acid (Brunswick, M. et al., J.J. J. Immunol. 140, 3364, 1988) or 6-bromohexanoic acid (Noguchi, A. et al., Bioconjugate Chem. 3, 132, 1992). The carboxylates may then be aminated with ethylene diamine to form an amine-terminal spacer and further reacted with amine-reactive heterobifunctional reagents to prepare dextran derivatives carrying terminal sulfhydryl residues, protected sulfhydryl residues such as thioester residues, or sulfhydryl-reactive groups such as pyridyl disulfide, maleimide, and iodoacetyl groups. A broad variety of useful amine-reactive heterobifunctional cross-linking reagents has been described most of which are commercially available (see e.g. Mattson, G. et al., Mol. Biol. Reports 17, 167, 1993).

In another embodiment of this invention, a polyaldehyde dextran has been further derivatized with homo- or heterobifunctional reagents containing a hydrazide residue on one end and a reactive residue suitable for this invention on the other end. For example, hydrazide-containing homobifunctional reagents such as carbohydrazide and adipic acid dihydrazide as well as hydrazide-containing heterobifunctional reagents such as the pyridyl disulfide-introducing reagent 3-(2-pyridyldithio) propionyl hydrazide (PDPH) (Zara, J.J. et al., Anal. Biochem. 194, 156, 1991) and the maleimide-introducing reagents 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH) (Chamow, S.M. et al., Bioconjugate Chem. 5, 133, 1994) and 4-(N-maleimido methyl) cyclohexane-1-carboxyl-hydrazide (M_2C_2H) may be conjugated to polyaldehyde dextran via hydrazone linkages. If further stabilization of the hydrazone linkage is required, sodium cyanoborohydride can be used to reduce the double bond and form a secure covalent linkage.

Thus, a wide range of dextran derivatives may be synthesized and utilized for derivatization of primer and detector oligonucleotides with multiple reactive residues.

VI.5. ENYZMATIC DETECTOR REAGENTS

In one preferred embodiment of this invention, enzymes are utilized as detector reagents. The catalytic activity of enzymatic detector reagents can be used to turn substrate molecules into chromogenic, fluorescent, chemiluminescent, bioluminescent,

or electrochemically detectable products. Such products are designated as reporter molecules.

VI.5.1. Preferred enzymes

Preferred examples of enzymes suitable for this invention are hydrolases (e.g., alkaline phosphatase, β-N-acetylglucosaminidase, arylsulfatase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, β-mannosidase, invertase, phosphodiesterase I, carboxypeptidase, carboxylic esterase, and β-lactamase) and oxidoreductases (e.g., glucose oxidase, xanthine oxidase, peroxidase, and glucose-6-phosphate dehydrogenase). In a more preferred embodiment, alkaline phosphatase (AP), horseradish peroxidase (HRP), β-galactosidase (β-Gal), and glucose oxidase (GO) are utilized as detector reagents for chromogenic, fluorescent, chemiluminescent, bioluminescent, and electrochemical assay procedures.

Alkaline phosphatases (orthophosphoric-monoester phosphorylase; EC 3.1.3.1) represent a large family of almost ubiquitous isoenzymes found in prokaryotic and eukaryotic organisms. In this invention, there is no restriction to specific members of this family. Thus, e.g. the two forms of alkaline phosphatase found in mammals may be used. They share common properties in that the phosphatase activity is optimal at pH 8 to 10, is activated by the presence of divalent cations, and is inhibited by cysteines, cyanides, arsenate, phosphate ions, and various metal chelators. The same applies to the calf intestinal enzyme having the highest catalytic rate constant (3500 s^{-1}) yet discovered for alkaline phosphatase isoenzymes. AP is capable of maintaining enzymatic activity for extended periods of substrate development. Therefore, increased sensitivity can be realized by extending the substrate incubation time to hours.

Horseradish peroxidase (donor:hydrogen peroxide oxidoreductase; EC 1.11.1.7) catalyzes the reaction of hydrogen peroxide with a variety of organic, electron-donating substrates. The reaction of HRP with H_2O_2 forms a stable intermediate that can dissociate in the presence of a suitable electron donor, thereby oxidizing the donor and creating an electrochemically or optically detectable reporter molecule. One important advantage of HRP is its robust nature and stability. HRP is a glycoprotein with a

molecular weight of 40 kDa. Its polysaccharide chains are often used in cross-linking reactions. Mild oxidation of the sugar residues with sodium periodate generates reactive aldehyde residues which may be used for conjugation to amine-containing molecules. The enzyme retains excellent activity after oxidation with sodium periodate.

β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) catalyzes the hydrolysis of β-D-galactoside in the presence of water to galactose and alcohol. β–Gal is composed of four identical subunits, each with an independent active site. The enzyme has divalent metals as cofactors. Chelated Mg^{2+} ions are required to maintain active site conformation. The presence of NaCl or dilute solutions (5%) of low molecular weight alcohols (e.g., methanol, ethanol) leads to enhanced substrate turnover. Mammalian β-galactosidases usually have a pH optimum within the range of 5.5 to 6, whereas β-galactosidase from *E. coli* has a pH optimum at 7 to 7.5. β-Gal is glycosylated and contains several sulfhydryl residues which may be utilized for cross-linking reactions.

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4) is a flavoenzyme that catalyzes the oxidation of β-D-glucose to δ-D-gluconolactone. The intermediate product of the catalysis is a reduced enzyme-$FADH_2$ complex that is oxidized back to enzyme-FAD in the presence of oxygen with the concomitant release of H_2O_2. The enzyme consists of identical subunits linked together by disulfide bonds. GO from *Aspergillus niger* has a pH optimum at 5.5, but can be used within the range of 4 to 7. In preferred assay procedures, the generated hydrogen peroxide is reacted with peroxidase and a suitable organic, electron-donating substrate. One particular advantage of the enzyme is the fact that there is no endogenous GO activity in mammalian tissues.

VI.5.2. Substrates for enzymatic detector reagents

A wide variety of substrates may be used to generate reporter molecules with enzymatic detector reagents of this invention. Suitable reporter molecules are those which may be detected using visual assessment, colorimetry, fluorometry, luminometry,

and electrometry. Also applicable for this invention are reporter molecules capable of initating recycling assay systems for amplified detection procedures.

VI.5.2.1. Reporter molecules for visual assessment

Qualitative and semiquantitative visual assessment procedures that are not dependent on instrumentation generate a change in color density and are carried out on paper strips or membranes. Examples of substrates suitable for this invention include a combination of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) for detector reagents with AP activity, and combinations of hydrogen peroxide and electron-donating compounds such as 4-chloro-napthol (4CN), 3,3',5,5'-tetramethylbenzidine (TMB), or 3,3'-diaminobezidine with or without nickel enhancement for detector reagents with HRP activity.

VI.5.2.2. Reporter molecules for colorimetric procedures

Examples of suitable substrates for colorimetric determinations are 4-nitrophenyl-phosphate for detector reagents with AP activity, 2-nitrophenyl-β-D-galactoside for detector reagents with β-Gal activity, and combinations of hydrogen peroxide and electron-donating compounds such as o-phenyl-diamine (OPD), 3,3',5,5'-tetramethylbenzidine (TMB), 3-methyl-2-benzothiazolinone hydrozone (MBTH), or (dimethylamino) benzoic acid (DMAB) for detector reagents with HRP activity. If desired, the procedure for monotoring of absorbance may be modified to extend the limited dynamic range of colorimetric measurements. The often limited dynamic range of colorimetric measurements can be extended by simultaneous monitoring of absorbance at two or more wavelengths, if applicable. For example, dual measurements of p-nitrophenol formed by alkaline phosphatase from p-nitrophenyl phosphate or of horseradish peroxidase and TMB as cosubstrat may be performed using wavelengths of 450 nm and 405 nm. This allows extension of the range of enzymatic activity readings by about a factor of five and three, respectively. Alternatively, the dynamic range of colorimetric measurements may be extended by means of kinetic recording techniques as opposed to fixed time measurements.

VI.5.2.3. Reporter molecules for fluorometric procedures

The use of fluorogenic substrates offers a dual advantage over chromogenic substrates in that the sensitivity of enzyme detection is significantly improved and the fluorometric detection affords a wider assay dynamic range than spectrophotometric detection. Examples of suitable substrates for fluorimetric determinations include 4-methylumbelliferyl phosphate (4MUP), 6,8-difluoro-4-methylumbelliferyl phosphate, fluorescein diphosphate, and dimethyl acridinone phosphate for detector reagents with AP activity, 4-methylumbelliferyl-β-D-galactopyranoside (4MUG), β–trifluoromethylumbelliferyl-β-D-galactopyranoside, 3-carboxyumbelliferyl-β-D-galactopyranoside, resorufin β-D-galactopyranoside, and fluorescein di-β–D-galactopyranoside for detector reagents with β-Gal activity, and combinations of hydrogen peroxide and electron-donating compounds such as p-hydroxyphenycarboxy acids (e.g., acetic, methoxyacetic, and propionic acid) for detector reagents with HRP activity. If desired, interference from contaminating fluorescent compounds is reduced by time-resolved fluorimetric measurements. Examples of suitable substrates for time-resolved fluorimetric determinations are 5-fluorosalicyl phosphate for detector reagents with AP activity. The product of the enzymatic hydrolysis, 5-fluorosalicylate, forms a highly fluorescent complex with Tb^{3+}-EDTA in alkaline solution. The ternary fluorescent complex absorbs at a wavelength that is characteristic of the chelator (337 nm) and emits fluorescence with a long fluorescence lifetime that is characteristic of Tb^{3+}.

VI.5.2.4. Reporter molecules for chemiluminometric procedures

Examples of suitable substrates for chemiluminescent detection of detector reagents with AP activity include adamantyl 1,2-dioxetane aryl phosphates such as disodium 3-(4-methoxyspirol [1,2-dioxetane-3,2'-tricyclo [3.3.1.1$^{3.7}$] decan-4-yl] phenyl-phosphate) (AMPPD) and the 5-chloro-substituted analog (CSPD). AP dephosphorylates these substrates to produce a phenoxide intermediate that generates light emission at 470 nm upon decomposition. The light emission from this reaction can be enhanced by certain polymers (e.g., polyvinylbenzyl (benzyldimethylammonium) chloride) and by detergent-fluorescein mixtures. Alternatively, detector reagents with AP activity may be quantitated by a chemiluminescent detection reaction using ascorbic acid 2-O-phosphate as substrate. Enzymatic cleavage of the phosphate group generates ascorbic acid which is quantitated under alkaline conditiions in the presence of oxygen

and lucigenin. Another chemiluminescent detection reaction of AP activity utilizes glucose-1-phosphate as substrate. Enzymatically generated glucose molecules are further reacted with glucose oxidase and the resulting hydrogen peroxide is quantitated using a mixture of microperoxidase and luminol. A related chemiluminescent detection reaction of AP activity utilizes 4-iodophenyl phosphate as substrate. Enzymatically generated 4-iodophenol is quantitated using horseradish peroxidase, hydrogen peroxide, and lunimol. Still another chemiluminescent detection reaction of AP activity utilizes $NADP^+$ as substrate and the resulting NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. In a subsequent reaction, NADH is quantitated using a mixture of microperoxidase, 1-methoxy-5-methylphenazinium methyl sulfate (1-MPMS), and luminol.

Examples of suitable substrates for chemiluminescent detection of detector reagents with β-Gal activity include but are not limited to disodium 3-(4-methoxyspirol [1,2-dioxetane-3,2'-tricyclo [3.3.1.1$^{3.7}$] decan-4-yl] phenyl-galactoside) (AMPGD). Enzymatic cleavage of the galactoside group from the 3-position of the aromatic ring of AMPGD produces the phenoxide derivative described above. Another chemiluminescent detection reaction utilizes lactose. Enzymatically generated glucose molecules are further reacted with glucose oxidase and the resulting hydrogen peroxide is quantitated using a mixture of microperoxidase and luminol, or a mixture of 8-anilino-1-naphthalenesulfonic acid (ANS) and bis (2,4,6-trichlorophenyl) oxalate (TCPO).

Examples of chemiluminescent assay procedures for detector reagents with glucose oxidase (GO) activity include those in which hydrogen peroxide generated by GO-mediated oxidation of glucose is quantitated using a mixture of microperoxidase and luminol, or a mixture of 8-anilino-1-naphthalenesulfonic acid (ANS) and bis (2,4,6-trichlorophenyl) oxalate (TCPO). These assay procedures can also be applied for chemiluminescent detection of detector reagents with glucose-6-phosphate dehydrogenase activity. Examples of chemiluminescent assay procedures for detector reagents with peroxidase (e.g., microperoxidase, HRP) activity include those in which luminol and related diacylhydrazides are utilized as cosubstrates.

If desired, the sensitivity of these assay procedures is increased by the addition of enhancer molecules such as substituted phenols (e.g., 4-iodophenol, 4-bromophenol, 4-hydroxycinnamic acid), naphthols (e.g., 1-bromonaphth-2-ol), amines (e.g., 4-methoxyaniline), and boronates (e.g., 4-iodophenylboronic acid). These molecules increase the light emission and reduce the assay reagent background which improves the signal to background ratio significantly.

Chemiluminescent assay procedures may also be utilized for detector reagents which exhibit enzymatic activities other than those mentioned above. Examples of such enzymatic activites include but are not limited to esterases, β-N-acetylglucosaminidase, invertase, xanthine oxidase, and urease. Detector reagents with esterase activity may be quantified using carboxyfluorescein diacetate as substrate. β-N-Acetylglucosaminidase may be quantified using the non-luminescent luminol-based substrate δ-aminophthalyl hydrazido-N-acetyl-β-D-glucosaminide. Invertase can be utilized to generate glucose from sucrose which may be quantified by GO-mediated oxidation reactions as described above. Xanthine oxidase may be assayed using a mixture of an iron-EDTA complex and luminol. A particular advantage of this assay is that the light emission from the xanthine oxidase-mediated chemiluminescent reaction is very long-lived (>96 hr).

VI.5.2.5. Reporter molecules for bioluminometric procedures

Examples of suitable substrates for bioluminescent detection of detector reagents with enzymatic activity include D-luciferin derivatives such as D-luciferin-O-phosphate for detector reagents with AP activity, D-luciferin-O-β-galactoside for detector reagents with β-Gal activity, D-luciferin-O-β-glucoside for detector reagents with β-glucosidase activity, D-luciferin-O-sulfate for detector reagents with arylsulfatase activity, D-luciferyl-L-Nα-arginine for detector reagents with carboxypeptidase B and N activities, D-luciferyl-L-phenylalanine for detector reagents with carboxypeptidase A activity, and D-luciferin methyl ester for detector reagents with carboxylic esterase activity. Released D-luciferin can be quantified by a luminometric detection assay using the bioluminescence system of *P. pyralis*.

VI.5.2.6. Reporter molecules for electrochemical procedures

In another preferred embodiment, electrochemical detection procedures are employed for determining the quantity of enzymatically generated reporter molecules. The electrochemical measurement of enzyme activity has a number of inherent advantages over optical detection systems. Variably cloudy or colored and even opaque solutions and containers can be monitored electrochemically.

Examples for reporter molecules for electrochemical detection procedures useful in the present invention include redox mediators which can be quantified via redox recycling using e.g. voltammetry in conjunction with a closely spaced array of thin film nobel metal electrodes. Preferred are interdigitated arrays (IDA) where anodes and cathodes have a width between 100 and about 800 nm and the electrodes are spaced apart from each other with a distance between 5 and 5000 nm, more prefably between 100 and about 800 nm. Examples of suitable substrates for electrochemical determinations using IDA microelectrodes include phosphomonoester derivatives of redox mediators for detector reagents with AP activity, phosphodiester derivatives of redox mediators for venom exonuclease, β-galactoside derivatives for detector reagents with β-Gal activity, β-glucoside derivatives for detector reagents with β-glucosidase activity, O-sulfate derivatives for detector reagents with arylsulfatase activity, L-phenylalanine derivatives for detector reagents with carboxypeptidase A activity, and methyl ester derivatives for detector reagents with carboxylic esterase activity. Prior to enzymatic action, preferred derivatives are essentially electrochemically not detectable, whereas after enzymatic cleavage they are detectable via redox recycling using a closely spaced arrray of thin film nobel metal microelectrodes. Therefore, useful redox mediators have structures that can be chemically derivatized to generate substrates with the desired properties. One example of preferred redox mediators is p-aminophenol which has been adjusted for the alkaline phosphatase amplification system by converting the reporter molecule to the electrochemically not detectable p-aminophenyl-phosphate.

Preferred redox mediators of this invention are further characterized by several additional properties. Both the electrochemically not detectable substrate and the

electrochemically detectable redox mediators should possess sufficient solubility in aqueous media to allow for a high substrate concentration and a good accessibility of generated redox mediator molecules to the micro electrodes. The redox potentials should exhibit reversible peaks in cyclic voltammograms and in aqueous solutions the potentials should be within the limits of - 600 mV (generation of oxygen) and + 800 mV (generation of hydrogen). Preferred are potentials close to 0 mV since they guarantee minimal background current as well as minmal instrumental background noise of the electrode, thereby providing optimal signal-to-noise ratios. Potentials close to 0 mV provide an additional advantage in that they minimize or avoid interference by electroactive compounds present in samples to be analyzed such as ascorbic acid in blood or catecholamines in urine.

One class of redox mediators contains one or more aromatic ring structures and various organic substituents and side chains that allow switching from a regular aromatic ring structure to a quinone structure and vice versa. Some examples of suitable redox mediators and their electrochemical properties are listed in Table I. The benzoquinone/hydoquinone couple and the p-aminophenol/quinoneimine couple are well-known redox couples of this class. Other examples are catechol and catechol derivatives (e.g., adrenalin, dihydroxyphenylalanine, epinine, adrenalone, norhomoepinephrine, and protocatechnic acid), dopamine, methoxytyraminin, aromatic compounds with more than one aromatic ring structure such as naphthol and anthracene derivatives, as well as heterocyclic aromatic compounds such as serotonin and hydroxyindolacetic acid. Aromatic redox mediators may be further derivatized with appropriate substituents to generate optimal redox potentials.

Table I. Examples of suitable redox mediators

Mediator	E_{anodic} [mV]	$E_{cathodic}$ [mV]	Collection Efficiency	Detection limit [nM]
p-aminophenol	350	- 150	0.85	5 - 10
o-hydroquinone	600	- 200	0.92	5 - 10
o-benzoquinone	600	- 200	0.92	5 - 10
dopamine	800	- 100	0.88	20
aderenalin	400	- 50	0.86	50
1-naphthol	400	0	0.90	5 - 10

VI.5.2.7. Recycling assay systems

Further, recycling assay systems may be employed for determining the quantity of enzymatically generated reporter molecules. Recycling systems for detector reagents with AP activity are e.g. those using the $NADP^+$ /NAD^+ system. NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. Subsequently, diaphorase catalyzes the reduction of another compound, thereby generating reporter molecules. Detector reagents with AP activity produce many NAD^+ molecules which are not consumed in the system but are recycled continuously, generating one reporter molecule per cycle. Useful chromogenic diaphorase substrates include but are not limited to p-iodonitrotetrazolium violet which forms an intensely purple formazan dye upon reduction. Useful electrometric diaphorase substrates for amperometric determinations include but are not limited to ferricyanide which is reduced by the diaphorase and reoxidized at the electrode. Useful fluorogenic diaphorase substrates include but are not limited to resazurin which forms the fluorescent compound resorufin upon reduction. The detection limit of multistage assay systems utilizing resazurin is below 500 molecules of AP.

VI.5.3. Derivatization of enzymatic detector reagents with reactive residues

Enzymatic detector reagents may be derivatized with reactive residues R2 suitable for covalent coupling to reactive residue-containing amplicons of target nucleic acids, reactive residue-containing detector oligonucleotides, or reactive residue-containing carrier molecules. This may be done by reacting an existing chemical group of the enzymatic detector reagent with a modification reagent that contains or produces the desired functional group. For example, an amine may be converted to a sulfhydryl or a carboxylate may be altered to yield an amine. This same type of modification strategy also can be used to create highly reactive residues from functional groups of rather low reactivity. For example, carbohydrate chains on enzymatic detector reagents may be modified with sodium periodate to transform rather unreactive hydroxyl groups into highly reactive aldehydes.

In one embodiment of the invention, enzymatic detector reagents lacking free sulfhydryl residues are chemically modified with sulfhydryl-introducing or sulfhydryl-creating reagents. Enzymatic detector reagents containing disulfide residues which are not essential for their functional activity may be selectively reduced to form free sulfhydryls. Alternatively, sulfhydryl residues may be introduced by reacting enzymatic detector reagents with amine-reactive, sulfhydryl-introducing reagents including but not limited to 2-iminothiolane (Jue, R. et al., Biochemistry 17, 5399, 1978), N-acetyl homocysteine thiolactone (Benesch, R., and Benesch, R.E. Proc. Natl. Acad. Sci. USA 44, 848, 1958), and SPDP (Carlsson, J. et al., Biochem. J. 173, 723, 1978). SPDP-derivatized assay component are further treated with dithiothreitol or another reducing agent to generate free sulfhydryl residues.

In another embodiment, enzymatic detector reagents are derivatized with protected reactive residues such as thioester groups, pyridyl disulfides, 2-nitrobenzoic acid disulfides, and sulfenylthiosulfate residues to avoid unwanted complex formation. Thioester residues may be introduced by reacting enzymatic detector reagents with amine-reactive, thioester-introducing reagents , e.g. SATA (Duncan, R.J.S. et al., Anal. Biochem. 132, 68, 1983), N-succinimidyl S-acetylthiopropionate (SATP) (Fuji, N. et al.,

Chem. Pharm. Bull. 33, 362, 1985), and SAMSA (Klotz, I.M., and Heiney, R.E. Arch. Biochem. Biophys. 96, 505, 1962). Pyridyl disulfide residues may be introduced by reacting enzymatic detector reagents with amine-reactive, pyridyl disulfide-introducing reagents including but not limited to SPDP (Carlsson, J. et al., Biochem. J. 173, 723, 1978). Alternatively, enzymatic detector reagents containing free sulfhydryl residues may be reacted with 4,4'-dipyridyl disulfide (Grasseti, D.R., and Murray, J.F. Arch. Biochem. Biophys. 119, 41, 1967) or 2,2'-pyridyl disulfide (Brocklehurst, K. et al., Meth. Enzymol. 34, 531, 1974). Alternatively, aldehyde-containing enzymatic detector reagents may be reacted with carbonyl-reactive, pyridyl disulfide-introducing reagents, e.g. PDPH (Zara, J.J. et al., Anal. Biochem. 194, 156, 1991). 2-Nitrobenzoic acid disulfides may be introduced by reacting enzymatic detector reagents containing free sulfhydryl residues with 5,5'-dithio-bis-(2-nitrobenzoic acid), Ellman's reagent (Ellman, G.L. Arch. Biochem. Biophys. 82, 70, 1959). Alternatively, enzymatic detector reagents containing free sulfhydryl residues may be derivatized with sulfenylthiosulfate residues by reaction with sodium tetrathionate (Pihl, A., and Lange, R. J. Biol. Chem. 237, 1356, 1962). Thus, a wide variety of reagents and methods is available for the derivatization of enzymatic detector reagents with suitable reactive residues, well-known to those skilled in the art.

VI.5.4. Use of non-derivatized enzymes as detector reagents.

Alternatively, non-derivatized enzyme molecules may be employed as detector reagents provided the reactive residues attached to detector oligonucleotides or carrier molecules are reactive with functional groups of non-derivatized enzyme molecules. Examples of residues reactive with functional groups of non-derivatized enzyme molecules such as sulfhydryls and amino groups include haloacetyl residues, maleimide residues, vinylsulfone residues, aldehyde groups, and reactive disulfide residues such as pyridyl disulfides, 2-nitrobenzoic acid disulfides, and sulfenylthiosulfate residues. For example, iodoacetyl residues can react with a number of functional groups within proteins including the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and the primary ε-amino group of lysine residues and N-terminal α-amines. The relative rate of reaction with each of these residues is dependent on the degree of ionization and thus the pH at

which the modification is performed. The exception to this is the methioninyl thioether that reacts rapidly at nearly all pH values above about 1.7. The relative reactivity of iodoacetyl residues toward protein functionalities is sulfhydryl > imidazolyl > thioether > amine. Utilizing these reactivities of iodoacetyl groups, assays can be designed which require a minimum of chemical derivatization of assay components.

VI.5.5. Immobilization of enzymes onto polymeric carrier molecules

As mentioned above, polymeric carrier molecules containing multiple immobilized enzyme molecules may be employed as detector reagents. Examples of polymers suitable as enzyme carriers include derivatives of polysaccharides, polyamino acids, polyvinyl alcohols, polyvinyl pyrrolidinones, polyacrylic acids, various polyurethanes, and polyphosphazenes. In a preferred embodiment, derivatives of dextran are employed as enzyme carriers. Preferred examples of suitable dextran derivatives include those described in section VI.4.2.1.

The methods by which enzymes may be covalently coupled to polymers are numerous and well-known in the art. For example, nucleophilic moieties of enzymes such as a primary amine, a thiol, or a hydroxyl group may be reacted with residues on polymeric carrier molecules that contain electrophilic moieties or have been derivatized with such a moiety. Examples of electrophilic moieties include, but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters, aldehydes, ketones, isothiocyano, maleimido, and carboxylic acid chloride residues. Thus, any of a wide range of functional groups on both the enzymes and the polymeric carrier molecules may be utilized for immobilization provided these groups are capable of forming covalent linkages.

One immobilization strategy involves the use of hetero- or homobifunctional cross-linking reagents. For example, enzymes may be derivatized with pyridyl disulfide groups (e.g., by reaction with SPDP) and subsequently coupled to sulfhydryl-containing polymeric carrier molecules via disulfide linkages. Remaining pyridyl disulfide residues which are not involved in the immobilization reaction can be utilized for covalent coupling of the enzyme-containing polymer to sulfhydryl groups on detector

oligonucleotdes or amplicons of target nucleic acids. Alternatively, the introduced pyridyl disulfide residues may be reduced with disulfide-reducing agents to create free sulfhydryl groups for coupling to sulfhydryl-reactive polymeric carrier molecules and to sulfhydryl-reactive residues on detector oligonucleotdes or amplicons of target nucleic acids. If SPDP should affect the enzymatic activity, there are a number of additional cross-linking reagents for coupling via disulfide bonds such as 2-iminothiolane (2-IT) SATA. 2-IT reacts with primary amines, instantly incorporating an unprotected sulfhydryl group. SATA also reacts with primary amines, but incorporates a protected sulfhydryl group, which is later deacetylated using neutral hydroxylamine to produce a free sulfhydryl group. Other cross-linkers are available that can be used in different strategies for immobilization of enzymes onto polymeric supports as well as for covalent coupling of enzyme-containing polymers to reactive residues on detector oligonucleotdes or amplicons of target nucleic acids. S-(2-Thiopyridyl)-L-cysteine hydrazide (TPCH) and S-(2-thiopyridyl) mercapto-propiono hydrazide (TPMPH) react with the carbohydrate moieties of glycosylated enzymes that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the cross-linking reagent and the periodate-generated aldehydes. TPCH and TPMPH introduce pyridyl disulfide residues which can be used for coupling reactions as described above. If disulfide bonding is unfavorable, other cross-linking reagents may be used. For example, GMBS and SMCC react with primary amines, thereby introducing a maleimide group for coupling to sulfhydryl-containing carrier molecules and detector oligonucleotides or amplicons of target nucleic acids via stable thioether linkages. Furthermore, cross-linking reagents may be used which introduce long spacer arms if steric hindrance problems interfere with the activity of the immobilized enzyme molecules. Thus, there is an abundance of suitable cross-linking reagents which could be used. Suitable reactions would be well-known to one skilled in the art based on the structural and functional characteristics of the enzyme to be immobilized and the nature of the reactive groups that are available or have been introduced.

VI.6. LIPOSOMAL DETECTOR REAGENTS

In another preferred embodiment of this invention, liposomes having reporter molecules encapsulated therein may be utilized as detector reagents. The surface of such liposomal detector reagents is derivatized with reactive residues R2 for covalent coupling to reactive residues on detector oligonucleotdes or amplicons on target nucleic acids. Preferred encapsulated reporter molecules may be a) chromogenic, fluorescent, and electrochemically detectable molecules, b) essential components of chemical detection systems such as non-enzymatic chemiluminescent reactions, c) essential, non-enzymatic components of enzymatic detection systems, d) enzyme activators, e) enzyme inhibitors, and f) enzymes capable of generating colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules. The release of reporter molecules from covalently coupled liposomes is effected e.g. by an increase of the ambient temperature or by the addition of liposome-lysing solvents such as organic solvents or detergents.

VI.6.1. Choice of liposomes

Liposomes are artificial structures primarily composed of phospholipid bilayers exhibiting amphiphilic properties. Other molecules, such as cholesterol, fatty acids, or lipid derivatives also may be included in the bilayer construction. The morphology of liposomes can be classified according to compartmentalization of aqueous regions between bilayer shells. If the aqueous regions are segregated by only one bilayer each, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV are further classified according to their relative size. Usually, the diameter of small unilamellar vesicles (SUV) is less than 100 nm with a minimum of about 25 nm, whereas the diameter of large unilamellar vesicles (LUV) is more than 100 nm with a maximum of about 2500 nm. MLVs typically form large complex honeycomb structures. As a consequence, of the almost infinite number of ways each bilayer can be associated and interconnected with next one, MLVs are difficult to categorize or exactly to reproduce. MLVs are the simplest to prepare, the most stable, and the easiest to scale up to large production levels. The most useful form of liposomes for this invention, however, consists of small, sherical ULVs

containing hydrophilic reporter molecules that are protected from the outer environment by the lipid shell. The outside surface is derivatized with reactive residues for covalent coupling to reactive residues on detector oligonucleotdes or amplicons of target nucleic acids.

VI.6.2. Methods for the preparation of liposomal detector reagents

Several methods are available to prepare liposomal detector reagents which are useful for this invention. On important group may be prepared using the following steps: (i) dissolving the lipid mixture in organic solvent, (ii) dispersion in an aqueous phase containing hydrophilic reporter molecules, and (iii) fractionation to isolate the correct liposomal population. During all handling procedures the solutions are preferably protected from excessive exposure to light. Organic solvents are preferably maintained under a nitrogen or argon atmosphere to prevent introduction of oxygen. Water and buffers are preferably degassed using a vacuum and bubbled with inert gas before lipid components are introduced.

In the first step of these methods, the lipid components are dissolved in organic solvent (e.g., chloroform: methanol, by volume 2:1). This mixture will include any phospholipid already derivatized with reactive residues or later to be derivatized, and/or other lipids used to form the liposomal structure. Once the desired mixture of lipid components is dissolved and homogenized in organic solvent, one of several techniques may be used to disperse the liposomes in aqueous solution containing the reporter molecules. Preferred methods include (i) mechanical dispersion, (ii) detergent-assisted solubilization, and (iii) solvent-mediated dispersion.

Using mechanical dispersion methods for the preparation of liposomal detector reagents, an aqueous solution containing the reporter molecules is added to the dried, homogeneous lipid mixture and manipulated to effect dispersion. For example, mechanical dispersion is possible, which may be performed by simple shaking, non-shaken aqueous contact, high-pressure emulsification, sonication, extrusion through small-pore membranes, and various freeze-thaw techniques. Most of these methods

result in a population of vesicles ranging from SUVs of only 25 nm diameter to very large MLVs.

Using detergent-assisted dispersion methods for the preparation of liposomal detector reagents, the amphipathic nature of detergent molecules is utilized to bring more effectively the lipid components into the aqueous phase for dispersion. The detergent molecules bind and mask the hydrophobic tails of lipids from the surrounding water molecules of the aqueous phase containing the reporter molecules. Detergent treatment may be performed using a dried lipid mixture or small vesicles. In a preferred embodiment, nonionic detergents such as the Triton X family, alkyl glycosides, or bile salts such as sodium deoxycholate are employed for this procedure. The immediate structures that form as the detergent molecules solubilize the lipids from a dried state are small micelles. On removal of the detergent from the solution, the lipid micelles aggregate to form larger liposome structures. Liposomes of up to 100 nm containing a single bilayer may be created using detergent-asssisted methods.

Using solvent-mediated dispersion techniques for the preparation of liposomal detector reagents, the lipid mixture is first dissolved in an organic solvent to create a homogeneous solution and thereafter introduced into an aqueous phase containing the reporter molecules. The solvent may or may not be soluble in the aqueous phase to effect this process. One preferred example of a solvent-mediated dispersion method is described by Batzri, S., and Korn, E.D. (Biochim. Biophys. Acta 298, 1015, 1973). Phospholipids and other lipids to be part of the liposomal membrane are first dissolved in ethanol. This ethanolic solution is then rapidly injected into a reporter molecule-containing aqueous solution of 0.16 M KCl using a Hamilton syringe, resulting in a maximum concentration of no more than 7.5% ethanol. Using this method, single bilayer liposomes of about 25 nm diameter can be formed. Other preferred solvent-mediated dispersion methods utilize solvents that are insoluble in the aqueous phase The production of liposomes by this procedure involves the formation of a 'water-in-oil' emulsion. To create a proper reverse-phase emulsion, a small quantity of aqueous phase containing the reporter molecules is introduced into a large quantity of organic phase containing the dissolved liposomes. The result is a milky dispersion. The

emulsification process involves the use of mechanical means (shaking, stirring, or sonication) to effect the formation of small droplets of aqueous solution uniformly dispersed in the lipid-organic phase. Excess of organic solvent is then removed by rotary evaporation (reverse-phase-evaporation method) until the mixture becomes a viscous gel. To facilitate liquification of the gel, a small volume of aqueous phase is added. Finally, residual organic solvent and untrapped reporter molecules are removed by dialysis.

VI.6.3. Choice of lipid components

Phospholipids are the most important constituents of liposomes serving as liposomal detector reagents. Two main forms of lipid derivatives exist biologically, molecules containing a glycerol backbone and those containing a sphingosine backbone. Naturally occurring phospholipids can be isolated from a variety of sources including egg yolk and may be used in the present invention. Preferred for this invention, however, is the use of synthetic phospholipids of known chemical purity. Three major fatty acid derivatives of synthetic phospholipids are used primarily for the preparation of liposomal detector reagents: (1) myristic acid (n-tetradecanoic acid; containing 14 carbons), (2) palmitic acid (n-hexadecanoic acid; containing 16 carbons), and (3) stearic acid (n-octadecanoic acid, containing 18 carbons).

Another significant component of liposomal detector reagents may be cholesterol. The addition of cholesterol to phospholipid bilayers alters the properties of the resultant membrane. The presence of cholesterol in liposome membranes has the effect of decreasing or even abolishing (at high cholesterol concentrations) the phase transition from gel state to the fluid or crystal state that occurs with increasing temperature. It also can modulate the permeability and fluidity of the associated membrane, increasing both parameters at temperatures below the phase transition point and decreasing both above the phase transition temperature.

VI.6.4. Choice of lipid composition

The ratio of lipid constituents is important to form stable liposomal detector reagents. Useful in the present invention is e.g. a liposomal composition (including phosphatidyl

ethanolamine (PE) derivatives) for encapsulating aqueous reporter molecules containing molar ratios of phosphatidyl choline (PC): cholesterol: negatively charged phospholipid (e.g., phosphatidyl glycerol, PG): derivatized PE of 8:10:1:1. Another useful composition using a maleimide derivative of PE without PG is PC: cholesterol: maleimide-PE of 85:50:15 (Friede, M. et al., Anal. Biochem. 211, 117, 1993). It may be preferred that the PE derivative does not exceed a concentration ratio of 1-10 mol PE per 100 mol of total lipid to maintain membrane stability.

Alternatively, liposomal detector reagents may be used which allow the release of encapsulated reporter molecules by a moderate increase of the ambient temperature. Liposomes are known to release encapsulated water-soluble contents more quickly near their liquid crystalline phase-transition temperature (T_m) than at other temperatures. Such a temperature-sensitive release can be engineered by the selection of pure lipids that undergo sharp transition temperatures or by using mutually miscible mixtures of pure lipids to adjust the transition temperature to the desired point. Examples of such lipids are dipalmitoyl phosphatidylcholine (DPPC) (T_m = 41°C), dipalmitoyl phosphatidyglycerol (DPPG) (T_m = 41°C), and distearoyl phosphatidylcholine (DSPC) (T_m = 54°C). The choice of lipids and the relative proportion of each depend upon the desired T_m and the size of the liposome. Small unilamellar vesicles (SUV) have apparent transition temperatures several degrees below those predicted from the T_m of the component lipids in large unilamellar vesicles (LUV) or in multilamellar vesicles (MLV). Typical lipid compositions of temperature-sensitive SUV, LUV, and MLV liposomes are known in the art, see e.g. Magin, R.L., and Weinstein, J.N. The design and characterization of temperature-sensitive liposomes. In: Liposome Technology, G. Gregoriadis, ed., vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984.

VI.6.5. Derivatization of liposomal detector reagents with reactive residues

Liposomal detector reagents useful for this invention contain surface-attached reactive residues R2 for covalent coupling to reactive residues on detector oligonucleotdes or amplicons of target nucleic acids as detailed above. If maleimides are selected, thay

may have adjacent stabilizing structures such as cyclohexane. In a preferred embodiment, reactive residues are utilized which allow long-term storage of liposomal detector reagents. Examples of such reactive residues are primary amines, hydrazides, pyridyl disulfides, 2-nitrobenzoic acid disulfides, halo acetyl residues, and thioester groups.

It is possible to use purified lipid components which are derivatized with reactive residues prior to incorporation into the lipid bilayer construction. Alternatively, the derivatization process of lipid components with reactive residues is performed after formation of the intact liposome. Numerous methods are available for derivatizing lipid functional groups with reactive residues, and preferred derivatization strategies include reductive amination, carbodiimide-mediated reactions, and heterobifunctional cross-linker-mediated derivatization reactions.

The most common type of heterobifunctional reagent for the activation of lipid components includes amine- and sulfhydryl-reactive cross-linking reagents containing an N-hydroxysuccinimide (NHS) ester on one end and a maleimide, iodoacetyl, or pyridyl disulfide group on the other end. Preferred examples of reagents for activation of lipid components include but are not limited to SMCC, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl-4-(p-maleimido phenyl) butyrate (SMPB), SIAB, SIAX, and SPDP (abbreviations see e.g. under VI.1.2.). Further, cross-linking reagents containing a spacer molecule may be used to activate lipid components. The presence of a spacer may be important in providing enough distance from the liposomal surface to ensure covalent binding of the liposomal detector reagent to reactive residues on captured amplicons of target nucleic acids or on hybridized detector oligonucleotdes. Examples of heterobifunctional cross-linking reagents with long spacer molecules (e.g., 6-aminohexanoate) include but are not limited to the long-chain versions of SPDP (LC-SPDP, succinimidyl 6-[3-(2-pyridyldithio)propionamido] hexanoate), and SIAX (SIAXX, succinimidyl 6-[6-{((iodoacetyl)amino)-hexanoyl}amino] hexanoate).

In one preferred embodiment, liposomes are used which are prepared using phosphatidyl ethanolamine (PE) of a synthetic variety having fatty acid constitutents of dimyristoyl (DMPE), dipalmitoyl (DPPE), or distearoyl (DSPE) forms, which is derivatized with heterobifunctional cross-linking reagents.Examples of heterobifunctional cross-linking reagents include but are not limited to those which derivatize the amino group of PE with a pyridyl disulfide group (e.g., by reaction with LC-SPDP), a maleimide residue (e.g., by reaction with SMPB), an iodoacetyl group (e.g., by reaction with SIAXX), a thioester moiety (e.g., by reaction with succinimidyl acetyl-thiopropionate; SATP), or an aldehyde function (e.g., by reaction with succinimidyl-p-formylphenoxyacetate; SFPA). In one preferred embodiment, the PE derivative is prepared before the liposome is constructed. Heterobifunctional reagents are preferred which are not modified with a sulfo residue at the N-hydroxysuccinimide ester moiety, since activation of PE is performed under non-aqueous conditions. Modification of PE prior to incorporation into liposomes offers an important advantage since a stable stock preparation of modified PE components can be made and used in a number of different liposome recipes. However, if reporter molecules to be entrapped within the liposome are reactive with the PE derivative, PE is activated after formation of the liposomal structures to ensure derivatization of only the outer half of the lipid bilayer. The sulfo-N-succinimidyl ester (sulfo-NHS) variety of the cross-linking reagents is preferred for activation of intact liposomes in aqueous suspension, since they are incapable of pentrating membranes.

VI.6.6. Reporter molecules of liposomal detector reagents

Useful reporter molecules for encapsulation in liposomal detector reagents are e.g. those described above.

VI.6.6.1. Liposome-encapsulated fluorescent reporter molecules

Fluorescent compounds may be utilized as reporter molecules which provide sufficient solubility in aqueous media to allow for efficient encapsulation in liposomal detector reagents. Examples of such fluorescent reporter molecules include fluorescein, cyanine dyes, lanthanide chelates, and phycobiliptroteins. Carboxyfluorescein is a frequently used fluorescent reporter molecule with a high quantum yield (0.85 in

alkaline aqueous solution) that has been encapsulated at high concentrations in liposomes (Locascio-Brown, L., et al., Clin. Chem. 39, 386,1993). Preferred examples of lanthanide ion chelates include but are not limited to chelates containing Eu^{3+}, Sm^{3+}, Dy^{3+}, and Tb^{3+}. Preferred chelating agents are strong metal chelators (formation constant $>10^{12}$ mol^{-1} liter) which are sufficiently soluble in aqueous media such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). Useful lanthanide ion chelates are exemplified by Tb^{3+} - EDTA chelates which form highly fluorescent complexes with 5-fluorosalicylate in alkaline solution. The phycobiliproteins B-phycoerythrin (B-PE), R-phycoerythrin (R-PE), and allophycocyanin (APC) are stable and represent highly soluble oligomeric proteins with quantum yields of up to 0.98. On a molar basis, the fluorescence yield is equivalent to at least 30 unquenched fluorescein or 100 rhodamine molecules at comparable wavelengths.

VI.6.6.2. Liposome-encapsulated electrochemically detectable reporter molecules

Electrochemically detectable compounds may be utilized as reporter molecules which are susceptible to redox recycling in a closely spaced array of thin film nobel metal electrodes. For details, see also VI.5.2.6 above because the same considerations apply. Preferred examples of such redox mediators are characterized by several important properties including sufficient solubility in aqueous media to allow for a high encapsulation efficiency in liposomal detector reagents and optimal accessibility of released redox mediator molecules to the microelectrodes.

One preferred class of such redox mediators contains one or more aromatic ring structures and various organic substituents and side chains that allow switching from a regular aromatic ring structure to a quinone structure and vice versa. Details see under VI.5.2.6.

Another preferred class of such redox mediators are organic and anorganic metal complexes that can be reversibly oxidized and reduced. In a more preferred embodiment, the metal complexes contain osmium, ruthenium, iron, copper, or chromium. Some of these metal complexes such as the ferricinium ion/ferrocene couple and bipyridyl derivatives of the Os(II)/Os(III) couple have been employed in

numerous amperometric and voltammetric assay procedures. Metal complexes provide several important advantages as redox mediators. Most important, metals can be complexed by various aromatic and heterocyclic aromatic compounds, the side chains of which may be used to adjust the redox potential according to the specific assay requirements. For example, the redox potential of ferrocene has been changed to both more anodic and more cathodic redox species by derivatization with residues such as carboxyl groups, halogens, aminoethyl groups, or pyridine derivatives. Preferred are electron positive side chains since they tend to change the potential of redox mediators towards O mV. Metal complexes provide a second important advantage in that they offer the possibility to introduce water-solubility by derivatization of the complexes with polar residues or groups that can be protonated. For example, covalent derivatization of the phenyl residues of Os-bipyridyl-complexes with carboxyl groups improved the water-solubility and, thereby, the access of theses complexes to the electrodes by a factor of 10 as compared to Os-bipyridyl-complexes formed with non-derivatized, hydrophobic phenyl residues. As a result, carboxyl-derivatized Os-bipyridyl-complexes provide both, high water-solubility and highly efficient redox reycling.

VI.6.6.3. Liposome-encapsulated components of non-enzymatic, chemiluminescent reactions

Components of non-enzymatic chemiluminescent reactions may be utilized as reporter molecules of liposomal detector reagents. Molecules with diverse structural features are capable of producing chemiluminescence. Some examples are shown in Table II. Preferred examples of components of non-enzymatic, chemiluminescent reactions are those which are sufficienty soluble in aqueous media to allow for efficient encapsulation in liposomal detector reagents.

Table II. Examples of non-enzymatic chemiluminescent reactions

Benzylamine	+	benzoyl chloride			
Benzylphenylketone	+	O_2			
Cyclopentanone	+	O_2			
Lithium diphenylphosphide	+	O_2			
Phenyl magnesium bromide	+	O_2			
Tetrakisdimethylaminoethylene	+	O_2			
Cysteine	+	O_2	+	Cu(II)	
Cyanomethyl	+	O_2	+	base	
Acridinium ester	+	peroxide	+	base	
Lophine	+	peroxide	+	base	
Lucigenin	+	peroxide	+	base	
Luminol	+	peroxide	+	base	
Oxalyl chloride	+	peroxide	+	anthracene	
Pyrogallol	+	peroxide	+	formaldehyde	
Tetrachlorethylidene carbonate	+	peroxide	+	fluorophore	
bis (2,4,6-Trichlorophenyl)oxalate	+	peroxide	+	fluorophore	
Rubrene	+	diphenyl peroxide			
Polystyrene	+	Dicyclohexyl peroxydicarbonate			
Zinc tetraphenylporphyrin	+	tetralin hydroperoxide			

VI.6.6.4. Liposome-encapsulated non-enzymatic components of enzymatic detection systems

Essential, non-enzymatic components of enzymatic detection systems may further be utilized as reporter molecules of liposomal detector reagents. Some examples of non-enzymatic components which are essential for certain enzymatic detection systems are those described in section VI.5.2. For example, light production by firefly luciferase (bioluminescence of *P. pyralis*) occurs via oxidation of D-luciferin in the presence of ATP and Mg^{2+} to oxyluciferin with emission of light at 546 nm. ATP can be encapsulated in liposomal detector reagents at high concentrations and, thereby, used

as reporter molecule for this bioluminescent detection system. NAD^+ (nicotinamide adenine dinucleotide) which also can be encapsulated in liposomal detector reagents at high concentrations, represents another useful example of essential, non-enzymatic components of enzymatic detection systems. NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. Generated NADH can be utilized in several diaphorase recycling assays as described in section VI.5.2.7.

VI.6.6.5. Liposome-encapsulated enzyme activators

In one preferred embodiment of the invention, activators of inactive enzymes are utilized as reporter molecules of liposomal detector reagents. Preferred encapsulated enzyme activators include metals for restoration of the catalytic activity of inactive apometalloenzymes, protein fragments for complementation of inactive deletion mutant enzymes, and enzyme subunits for activation of incomplete enzyme complexes.

Metalloenzymes useful in this connection are e.g. metallophosphatases (e.g., alkaline phosphatase from *Escherichia coli*), metallodehydrogenases (e.g., alcohol dehydrogenase from horse liver), and metalloproteases (e.g., aminopeptidase M from pig kidney). Table III gives examples of metalloenzymes from which inactive apoenzymes have been prepared. The intrinsic metal atoms of such metalloenzymes can be removed by treatment with appropriate chelating agents and replaced completely with corresponding loss and restoration of catalytic activity. Metals encapsulated in affinity liposomes may also be utilized as buffer constitutents to mediate catalytic activity of non-metalloenzymes which have an absolute requirement for certain metals. Examples of such enzymes are venom exonuclease (phosphodiesterase I) from *Crotalus adamanteus* which has an absolute requirement for Mg^{2+} and is completely inhibited by treatment with the chelating agent EDTA.

Table III. Examples of metalloenzymes from which inactive apoenzymes have been prepared (Wagner, F.W. Meth. Enzymol. 158, 21, 1988)

Enzyme	Number of subunits	Metal/subunit	Chelating agent used to prepare apoenzyme
Alkaline Phosphatase (*E. coli*)	2	2 Zn^{2+}, 1 Mg^{2+}	1,10-phenanthroline, EDTA, Chelex 100
Aminopeptidase (*Aeromonas*)	1	2 Zn^{2+}	1,10-phenanthroline
Aminopeptidase (bovine lens)	6	2 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase A	1	1 Zn^{2+}	1,10-phenanthroline
Procarboxy-peptidase A	1	1 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase B	1	1 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase (*Steptomyces griseus*)	1	1 Zn^{2+}	1,10-phenanthroline
Neutral protease (*Bacillus subtilis*)	1	1 Zn^{2+}	EDTA
Thermolysin	1	1 Zn^{2+}, 1 Ca^{2+}	1,10-phenanthroline
Angiotensin converting enzyme	1	1 Zn^{2+}	1,10- phenanthroline
Alcohol dehydroge-nase (horse liver)	2	2 Zn^{2+}	dipicolinic acid
Carbonate dehydratase	1	1 Zn^{2+}	1,10-phenanthroline, dipicolinic acid
Superoxide dismutase dismutase	2	2 Cu^{2+},1 Zn^{2+}	EDTA

Preferred encapsulated enzyme activators may also be polypeptides for complementation of inactive deletion mutant enzymes. Examples are complementation of the inactive deletion mutant M15 (α-acceptor) of β-galactosidase from *Escherichia coli* by the 90-residue peptide CB2 which restores the activity of the M15 mutant to approximately two-thirds of the level of native β-galactosidase.

VI.6.6.6. Liposome-encapsulated enzyme inhibitors

Inhibitors of enzymatic detection systems may also be utilized as reporter molecules of liposomal detector reagents. A detailed review of inhibitors for various enzymatic detector reagents is provided by Haugland, R.P. (In: Handbook of fluorescent probes and research chemicals (M-T.Z. Spence, ed.), pp. 202-243, Molecular Probes, Inc., Eugene, OR, USA, 1996). Several considerations are important for the choice of enzyme inhibitors suitable for use in the present invention. Sufficient solubility in aqueous solutions, low molecular weight, and high affinity binding of the inhibitor to the corresponding enzyme are the most important requirements. Preferred overall binding constants (K_{off}/K_{on}) are in the low nanomolar to picomolar range to guarantee efficient inhibition. Methotrexate represents one example of such an inhibitor. Methotrexate is a water-soluble compound with a molecular weight of 508.5 daltons, and binds to dihydrofolate reductase (DHFR) with an overall binding constant of 2.1×10^{-10} M. DHFR oxidizes NADH which can be monitored at 340 nm.

Although small molecular weight inhibitors are preferred, high molecular weight inhibitors are also useful in this invention. For example, the placental ribonucleae inhibitor (RPI) is a 50kD protein that forms tight complexes with ribonucleases RPI inhibits RNase A with an extremely low K_i value of 4×10^{-14} M, approaching the affinity of avidin for biotin.

VI.6.6.7. Liposome-encapsulated enzymes

In another preferred embodiment, enzymes capable of generating colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules are utilized as reporter molecules of liposomal detector reagents. Examples of enzymes useful for this application include those described in section VI.5.1.

VI.6.7. Encapsulation and release of reporter molecules

The encapsulation efficiency of water-soluble reporter molecules within liposomal detector reagents depends on the liposome type. A comparative analysis of the encapsulation effiency in SUVs, LUVs, and MLVs of the water-soluble marker

compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) has been performed by Magin, R.L., and Weinstein, J.N. In: Liposome Technology (G. Gregoriadis, ed.), vol. IV., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Typical values of this study are given in Table IV below. For a fixed quantity of lipid, LUVs have the highest encapsulation efficiency.

Table IV. Encapsulation characteristics of different types of liposomes

	LIPOSOME TYPE		
	SUV (DPPC:DSPC) (7:3)	LUV (DPPC:DPPG) (4:1)	MLV (DPPC:DPPG) (4:1)
Diameter (nm)	20 - 50	70 - 800	125 - 2000
Captured volume (l/mg lipid)	0.1 - 0.6	7 - 12	5 - 7
Encapsulation efficiency (%)	0.2 - 2.0	20 - 40	15 - 20

DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidyglycerol; DSPC, distearoyl phosphatidylcholine

In a preferred embodiment, the release of encapsulated reporter moecules is mediated by the addition of detergent (i.e., addition of Triton X-100 to a final concentration of 0.4%, or sodium deoxycholate to a final concentration of 0.05 - 0.2%), organic solvent, or by an increase of the ambient temperature (phase-transition release). Typical values obtained with the marker compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) are summarized in Table V. Preferred heating rates at passage through T_m are 10 to 15°C/min. Based on these data, detergent-mediated release of encapsulated reporter molecules represents the choice of release mechanism when ultimate detection sensitivity is required. The addition of detergent, however, requires an additional container, whereas heating to approximately

45°C is relatively easy to accomplish. Therefore, phase-transition-mediated release represents the choice of release mechanism for less sensitive but for more cost-efficient assay procedures.

Table V. Release characteristics of different types of temperature-sensitive liposomes

	LIPOSOME TYPE		
	SUV (DPPC:DSPC) (7:3)	LUV (DPPC:DPPG) (4:1)	MLV (DPPC:DPPG) (4:1)
Diameter (nm)	20 - 50	70 - 800	125 - 2000
Release (%) (1 min at 42°C)	5 - 10	30 - 60	30 - 60

DPPC, dipalmitoyl phosphatidylcholine (T_m = 41°C); DPPG, dipalmitoyl phosphatidyglycerol (T_m = 41°C); DSPC, distearoyl phosphatidylcholine (T_m = 54°C)

VI.6.8. Storage of liposomal detector reagents

The most useful form of liposomal detector reagents for this invention consists of small, spherical ULVs with surface-attached reactive residues and entrapped hydrophilic reporter molecules. Small liposome vehicles, however, often aggregate on standing to form larger, more complex structures. In a preferred embodiment, cryoprotectants such as sugars or polyhydroxylic compounds are employed to prevent structural degradation problems on freezing. The hydroxyl groups in cryoprotectants can take the place of water in hydrogen bonding activities, thus providing structural support even under conditions in which water is removed.

VI.7. SIGNAL AMPLIFICATION USING ADDITIONAL POLYMERIC CARRIER MOLECULES

Sensivity of the detection and/or quantification of nucleic acids according to the basic embodiments of the present invention will be sufficient for many cases. However, for applications that require higher detection sensitivity, or at least wherein such a higher detection sensitivity is desired, an amplification step may be required. In some embodiments of the present invention, additional polymeric carrier molecules are added to the system. For example, captured target nucleic acids are detected by reactive residue(R1)-containing detector oligonucleotides, or captured amplicons of target nucleic acids are generated with reactive residue(R1)-containing primer oligonucleotides, respectively, followed by covalent binding of hydrophilic polymeric carrier molecules containing two different types of reactive residues, one or more type I reactive residues R3 capable of covalent binding to reactive residues R1, and multiple (i.e. at least two) type II reactive residues R3 capable of covalent binding multiple detector reagents (see e.g. Fig. 5). The type I reactive residues may be the same as the type II reactive residues, or they may be different, depending on the reactive residues used in the detector oligonucleotides and those used in the detector reagents or primer oligonucleotides, respectively. As a result of covalent binding of multiple detector reagents, the signal may be amplified by one to two orders of magnitude.

Preferred examples of synthetic and natural polymeric carrier molecules are derivatives of polysaccharides, polyamino acids, polyvinyl alcohols, polyvinyl pyrrolidinones, polyacrylic acids, various polyurethanes, polyphosphazenes, and copolymers of these polymers. In a more preferred embodiment, derivatives of dextran are employed as polymeric carrier molecules. Preferred examples of suitable dextran derivatives include those described in section VI.4.2.1. Reactive residues suitable for derivatization of additional polymeric carrier molecules include those described in section VI.4.1.

VI.8. SIGNAL AMPLIFICATION USING COVALENTLY LINKED COMPLEXES OF DETECTOR REAGENTS

In another preferred embodiment of enhanced covalent amplification systems, covalently linked complexes of detector reagents may be used. Such covalently linked

complexes can be formed by chemical reaction of two types of detector reagents containing different reactive residues. The reactive residues R2 of type I detector reagents are capable of covalent binding to reactive residue-containing detector oligonucleotides or to reactive residue-containing amplicons of target nucleic acids, and the reactive residues R4 of type II detector reagents are capable of forming covalent linkages with type I detector reagents. Alternatively, covalently linked complexes of detector reagents may be formed by chemical reaction of one type of detector reagent containing reactive residues capable of both, covalent binding to reactive residue-containing detector oligonucleotides or to reactive residue-containing amplicons of target nucleic acids, respectively, and forming covalent linkages with homobifunctional cross-linking reagents or reactive residue-containing polymers, or directly with other reactive residues of the same detector reagent type.

Cross-linked detector reagents may be employed as preformed complexes in enhanced covalent amplification assays. Preferred cross-linked detector reagents are covalently linked complexes of derivatized enzyme molecules or liposomes containing encapsulated reporter molecules and surface-attached reactive residues. Preformed complexes of detector reagents may be prepared by coupling procedures with additional spacer molecules. Especially preformed enzymes complexes may require additional spacer molecules to retain enzymatic activity. Useful spacer molecules are e.g. oligoethylene derivatives (for a review, see Hermanson, G.T. (ed.) Bioconjugate Techniques. Academic Press, San Diego, 1996) and the broad variety of homo- and heterobifunctional cross-linking reagents most of which are commercially available (for a review, see Mattson et al., Mol. Biol. Reports 17, 167, 1993).

VI.8.1. Synthesis of covalently linked enzyme complexes

Enzyme molecules may be derivatized with amine-reactive heterobifunctional cross-linking reagents prior to complex formation. For example, one part of enzyme molecules may be derivatized with pyridyl disulfide residues by reaction with SPDP and the other part with free sulfhydryl groups by reaction with 2-iminothiolane. Using appropriate derivatization conditions, each enzyme molecule will be derivatized with a few reactive residues. Upon mixing of these two enzyme preparations, complexes will form via thiol-

disulfide exchange. If disulfide bonding is found unsuitable for producing stable complexes, other cross-linking reagents may be used that form more stable bonds. For example, the heterobifunctional cross-linker N-γ-maleimido butyryloxy succinimide (GMBS) reacts with primary amines, thus introducing a maleimide group. This maleimide group can subsequently react with a free sulfhydryl group, thereby forming a stable thioether linkage. If steric hindrance problems have to be solved, cross-linkers such as a long-chain version of SPDP can be used which introduce long spacer arms between the cross-linked enzyme molecules.

Alternatively, covalently linked complexes of enzymes may be formed by chemical reaction of one type of enzyme or enzyme derivative using homobifunctional cross-linking reagents or polymers containing reactive residues capable of forming a covalent linkage with the detector reagent. For example, enzyme molecules derivatized with pyridyl disulfide residues (e.g., by reaction with SPDP) may be cross-linked by homobifunctional spacer molecules containing two sulfhydryl residues. Enzyme molecules derivatized with free sulfhydryl residues (e.g., by reaction with 2-iminothiolane) may be cross-linked by homobifunctional sulfhydryl-reactive cross-linking reagents such as 1,4-di-[3'-(2'-pyridyldithio) propionamido] butane (DPDPB) and bis-maleimidohexane (BMH). The skilled person is aware of an abundance of suitable cross-linking reagents. Considerations for suitably selected reactions are based on the well known structural and functional characteristics of the enzyme to be cross-linked and the nature of the reactive groups that are available or have been introduced.

VI.8.2. Synthesis of covalently linked liposome complexes

Covalently linked complexes of liposomal detector reagents are synthesized as described for covalently linked enzyme complexes (section VI.8.1.). In one preferred embodiment, two types of liposomal detector reagents are used for complex formation. For example, liposomal detector reagent type I may be derivatized with pyridyl disulfide residues and liposomal detector reagent type II with free sulfhydryl residues. Upon mixing of these two preparations of liposomal detector reagents, complexes will form via thiol-disulfide exchange. If disulfide bonding is found unsuitable for producing stable complexes, other reactive residues may be used that form more stable bonds. For

example, the reaction of maleimide groups with free sulfhydryl groups leads to the formation of stable thioether linkages. If required, long spacer arms between the surface of the liposomal detector reagents and the reactive residues can be used.

Alternatively, covalently linked complexes of liposomal detector reagents may be be formed by chemical reaction of one type of liposomal detector reagent using homobifunctional cross-linking reagents or polymers containing reactive residues capable of forming a covalent linkage with the liposomal detector reagent. For example, liposomal detector reagents derivatized with pyridyl disulfide residues may be cross-linked by homobifunctional spacer molecules containing two sulfhydryl residues. Liposomal detector reagents derivatized with free sulfhydryl residues may be cross-linked by homobifunctional sulfhydryl-reactive cross-linking reagents such as those mentioned under VI.8.1. Thus, a variety of methods well-known to one skilled in the art is available for the synthesis of covalently linked complexes of liposomal detector reagents.

VII. EXAMPLES

VII.1. ACTIVATION OF OLIGONUCLEOTIDES

Activation of oligonucleotides is required for immobilization of capture oligonucleotides and the synthesis of reactive residue-containing primer and detector oligonucleotides.

VII.1.1. Synthesis of 5'-amine derivatives of oligonucleotides

Method A. Covalent attachment of an amine terminal spacer molecule to the 5'-phosphate of oligonucleotides according to method A is performed via formation of a phosphorimidazolide intermediate in a carbodiimide reaction (based on the method of Ghosh, S.S. et al., Anal. Biochem. 178, 43, 1989). The formation of a phosphoimidazolide intermediate provides better reactivity towards amine nucleophiles than the carbodiimide phosphosdiester intermediate if carbodimide is used without added imidazole. The carbodiimide phosphodiester intermediate also is shorter-lived in aqueous conditions due to hydrolysis than the imidazolide.

The 5'-phosphate-containing oligonucleotide (7.5 -15 nmol in 7.5 μl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA). Immediately, 5 μl of 0.25 M bis-hydrazide compound (e.g., carbohydrazide or adipic acid dihydrazide) dissolved in 0.1 M imidazole, pH 6, is added. The reaction mixture is vortexed and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 μl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. The hydrazide-labeled oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

For derivatization of 5'-phosphate-containing oligonucleotides with diamine spacer molecules (e.g., ethylene diamine or 1,6-diaminohexane) the same experimental procedue is used. The diamine compound is dissolved at a concentration of 0.25 M in 0.1 M imidazole, pH 6, and 5 μl of this soluition is added to the reaction mixture.

Method B. Using method B, the desired oligonucleotide is prepared using automated standard solid-phase phosphoramidite techniques on a scale of about 1 μmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of N-monomethoxytrityl O-methoxydiiso propylaminophosphinyl 3-aminopropan (1) ol (prepared according to Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987) and 60 μmol of tetrazole. Thereafter, the methyl phosphate protecting groups are removed with thiophenol and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (25% B at t = 0 min ; 75% B at t = 20 min). After

removal of the solvent by evaporation, the purified monomethoxytrityl protected oligonucleotide is dissolved in 2 ml of 80% acetic acid and incubated for two hours. Thereafter, the acetic acid is removed by evaporation and the detritylated amino-containing oligonucleotide is is redissolved in a small volume of water.

VII.1.2. Synthesis of 5'-sulfhydryl derivatives of oligonucleotides

Method A. For the derivatization of 5'-phosphate-containing oligonucleotides with a terminal sulfhydryl group according to method A (based on a procedure of Ghosh, S.S., Kao, P.M., and Kwoh, D.Y. Bioconjugate Chem. 1, 71, 1990), the oligonucleotide (7.5 - 15 nmol in 7.5 µl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA). Immediately, 5 µl of 0.25 M cystamine dissolved in 0.1 M imidazole, pH 6, is added. The reaction volume is mixed by vortexing and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 µl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. For reduction of the cystamine-derivatized oligonucleotide, 20 µl of 1 M dithiothreitol is added. Thereby, 2-mercaptoethylamine is released from the cystamine modification site and a terminal free sulfhydryl group is created at the 5'-position of the oligonucleotide. After 15 min at room temperature, the sulfhydryl-derivatized oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

Method B. Using method B, the desired oligonucleotide is prepared by automated standard solid-phase phosphoramidite techniques on a scale of about 2.5 µmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 µmol of an S-trityl-O-methoxymorpholinophosphite derivative of 2-mercaptoethanol (dissolved in 0.3 ml of acetonitile and 0.2 ml of 1,2-dichloroethane), 3-mercaptopropan (1) ol (dissolved in 0.5 ml of acetonitrile), or 6-mercaptohexan (1) ol

(dissolved in 0.5 ml of acetonitrile) and 75 μmol of tetrazole (dissolved in 0.5 ml of acetonitrile). The S-trityl-O-methoxymorpholinophosphite derivatives are prepared according to Connolly, B.A., and Rider, P. (Nucleic Acids Res. 13, 4485, 1985). Following coupling, the phosphite intermediate is oxidized by treatment with iodine. Thereafter, the phosphate protecting groups are removed with thiophenolate and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (10% B at t = 0 min ; 80% B at t = 30 min; 100%B at t = 40 min). The purified S-trityl-containing oligonucleotide in 0.1 M triethylammonium acetate, pH 6.5, is treated with a five-fold molar excess of $AgNO_3$. After 30 min a seven-fold molar excess of dithiothreitol is added and after another 30 min the precipitated Ag^+ salt of dithiothreitol is removed by centrifugation. After reduction of the volume by rotary evaporation, the thiol-containing oligonucleotide is used immediately for further derivatization. Alternatively, the thiol-containing oligonucleotide can be stored frozen at -20 °C for months with no decomposition.

VII.1.3. Pyridyl disulfide modification of 5'-amine-containing oligonucleotides

Oligonucleotides that have been modified with an amine-terminal spacer molecule can be reacted further with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA). Oligonucleotides derivatized with a terminal pyridyl disulfide residue then can be coupled with sulfhydryl-containing molecules, forming a disulfide bond. Reduction of the terminal pyridyl disulfide residue releases the pyridine-2-thione leaving group and generates a terminal sulhydryl group. This procedure allows conjugation of the 5'-thiolated oligonucleotide to sulfhydryl-reactive derivatives.

First, SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). The amine-derivatized oligonucleotide is dissolved in 250 μl of 50 mM

sodium phosphate, pH 7.5, and mixed with 50 µl of the SPDP solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

To release the pyridine-2-thione leaving group and form the free sulfhydryl, the oligonucleotide derivative is mixed with 20 µl of 1 M dithiothreitol and incubated for 15 min at room temperature. If present in sufficient quantity, the release of pyridine-2-thione can be monitored by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$). The thiolated oligonucleotide is purified from ecxess dithiothreitol by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The thiolated oligonucleotide is used immediately for further coupling reactions to prevent sulfhydryl oxidation.

VII.1.4. Thioester modification of 5'-amine-containing oligonucleotides

The NHS ester of SATA (Pierce Chemical Company, Rockford, IL, USA) introduces a thioester moiety. The acetyl protecting group can be removed by treatment with neutral hydroxylamine. The resulting terminal sulfhydryl group can be used for subsequent *conjugation to thiol-reactive molecules. The advantage of using* SATA *over disulfide-*containing thiolation reagents such as SPDP is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. The pyridiyl dithiol group resulting from an SPDP thiolation must be reduced with a reducing agent such as dithiothreitol to free the sulfhydryl group. With SATA, the sulfhydryl is freed by hydroxylamine, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

First, SATA is dissolved at a concentration of 8 mg/ml in DMF. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 250 µl of the SATA solution. After reaction for 3 hours at 37 °C, excess reagents are removed from the modified oligonucleotide by gel filtration. To deprotect the thioacetyl group, 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, is added and reacted for 2 hours at 37 °C. The sulfhydryl-containing oligonucleotide is used immediately for further reaction with a sulfhydryl-reactive molecule.

VII.1.5. Derivatization of oligonucleotides with an aldehyde function at the 5'-terminus

The cross-linking reagent SFB (succinimidyl *p*-formylbenzoate) can be used to add aldehyde groups to amine-containing oligonucleotides. First, SFB is dissolved at a concentration of 12.35 mg/ml in acetonitrile (makes a 50 mM solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SFB solution. After reaction for 3 hours at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VII.2. COUPLING OF ACTIVATED OLIGONUCLEOTIDES TO SPACER MOLECULES

To increase the flexibility and, thereby, the reactivity of immobilized capture oligonucleotides, spacer molecules may be introduced between the solid support and the immobilized capture oligonucleotides. In order to avoid potential steric hindrance problems, detector or primer oligonucleotides containing terminal attached oligomeric or polymeric carrier molecules with multiple reactive residues may also require a spacer molecule between the oligonucleotide and the reactive residue-containing carrier molecule.

Method A. In method A, the heterobifunctional reagents LC-SPDP or sulfo-LC-SPDP (both Pierce Chemical Company, Rockford, IL, USA) are utilized as spacer molecules. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that provides water-solubility to the cross-linker.

LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ ml water. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5,

and mixed with 50 µl of the LC-SPDP solution. If the water-soluble sulfo-LC-SPDP is used, 100 µl of the sulfo-LC-SPDP solution is added. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

Method B. In method B, water-soluble heterobifunctional derivatives of polyethylene glycol (PEG) are utilized as spacer molecules. Heterobifunctional PEG derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety are especially useful, since the VS moiety is hydrolytically stable in aqueous media. At pH 7, the VS moiety reacts selectively with sulfhydryl groups. Reaction with amino groups proceeds at higher pH, but is still relatively slow.

NHS-PEG-VS (MW 3400) or NHS-PEG-VS (MW 2000); both Shearwater Polymers Europe, Enschede, Netherlands, is dissolved in DMF at a concentration of 10 mM. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 100 µl of the NHS-PEG-VS solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VII.3. IMMOBILIZATION OF ACTIVATED CAPTURE OLIGONUCLEOTIDES

For the immobilization of activated capture oligodeoxynucleotides, a variety of solid support systems providing different reactive residues is available. The examples describe the preparation of some suitable solid support systems and their application for immobilization of activated capture oligodeoxynucleotides. Each of the described procedures can be easily adjusted for the introduction of spacer molecules between the solid support and the immobilized capture oligonucleotides to increase the flexibility and, thereby, the reactivity of immobilized capture oligodeoxynucleotides. Furthermore, each of the described procedures allows to modify the density of immobilized capture oligonucleotides by co-immobilizing hydrophilic compounds capable of blocking immobilization sites for capture oligodeoxynucleotides (e.g., for amine-reactive solid

supports: aminoethanol or monomethoxy-poly(ethylene glycol) containing a terminal amino group).

VII.3.1. Immobilization of activated capture oligodeoxynucleotides onto mercapto-activated beaded agarose

The preparation of mercapto-activated cross-linked agarose beads is performed as described by Porath, J., and Axen, R. (Meth. Enzymol. 44, 19, 1976). Sepharose 6B (30 g of moist cake) is washed with water, suction-dried, suspended in 24 ml of 1 M NaOH followed by dropwise addition of epichlorohydrin over 15 min at room temperature with stirring (0.75 ml of epichlorohydrin is added for a degree of subsitution of approximately 50 μmol of SH-groups per gram beads; 4.5 ml of epichlorohydrin is added for a degree of subsitution of approximately 700 μmol of SH-groups per gram beads). The suspension is then incubated with continuous stirring for 2 hrs at 60°C. The product is washed with water and with 0.5 M sodium phosphate buffer, pH 6.25. After suction-drying and resuspension in 30 ml of 0.5 M sodium phosphate buffer, pH 6.25, 30 ml of 2 M sodium thiosulfate are added to the beads, followed by stirring for 6 hrs at room temperature. The alkyl thiosulfate ester obtained is washed with water, suspended in 60 ml of 0.1 M sodium bicarbonate, and reduced by the addition of 50 ml of dithiothreitol (8 mg/ml) containing 1 mM EDTA. The resulting mercapto beads are washed with 300 ml of 0.1 M sodium bicarbonate, 1.0 M NaCl, 1 mM EDTA, and then with 100 ml of 1 mM EDTA. The mercapto beads are stored in 10 mM deaerated sodium acetate, 1 mM EDTA, pH 4.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the mercapto-activated cross-linked agarose beads are filtered and washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VII.3.2. Immobilization of activated capture oligodeoxynucleotides onto pyridyl disulfide- activated beaded agarose

Mercapto-activated cross-linked agarose beads (section VII.3.1) are washed with water and a solution of 50% acetone-water. Thereafter, the beads are resuspended in 50% aqueous acetone and mixed with 100 mg 2,2'-dipyridyl disulfide dissolved in 50% aqueous acetone. After 30 min at room temperature, the beads are washed with 50% aqueous acetone containing 1 mM EDTA.

Prior to coupling of 5'-sulfhydryl-derivatized oligodeoxynucleotides, the pyridyl disulfide-activated cross-linked agarose beads are filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-sulfhydryl-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water.

VII.3.3. Immobilization of activated capture oligodeoxynucleotides onto carbonyldiimidazole-activated beaded agarose

The preparation of carbonyldiimidazole (CDI)-activated cross-linked beaded agarose is performed as described by Wilchek, M. et al. (Meth. Enzymol. 104, 3, 1984). Cross-linked Sepharose 6B (3 g of moist cake) is washed sequentially with 20 ml each of water, dioxane-water (3: 7), dioxane-water (7: 3), and dioxane and ist suspended in 5 ml of dioxane. 1,1'-Carbonyldiimidazole (120 mg) is added, and the suspension is shaken at room temperature. After 15 min, the suspension is washed with 100 ml dioxane and used immediately. Alternatively, the matrix may be stored in dioxane under anhydrous conditions.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the CDI-activated cross-linked agarose beads are filtered and washed quickly with 0.1 M sodium bicarbonate, pH 8.5. Thereafter, the moist beads are resuspended in 0.1 M sodium bicarbonate, pH 8.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the residue is filtered and

washed with 0.1 M $NaHCO_3$ and with distilled water. Excess active groups are removed by reaction for 1 hr at room temperature with either 0.1 M NH_4OH or 0.1 M ethanolamine at pH 9.

VII.3.4. Immobilization of activated capture oligodeoxynucleotides onto tresyl-activated beaded agarose

The preparation of tresylated cross-linked beaded agarose is performed as described by Nilsson, K., and Mosbach, K. (Meth. Enzymol. 104, 56, 1984). Sepharose 4B (10 g, wet) is washed successively with 100 ml of each of the following: 30: 70 and 70: 30 of acetone: water (v/v), twice with acetone, and three times with dry acetone (dried with a molecular sieve overnight) using 1 liter of acetone per 35 g of Sepharose 4B. The gel is then transferred to a dried beaker containing 3 ml of dry acetone and 150 µl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 100 µl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 10 min at room temperature, the gel is washed twice with 100 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone, 70: 30 of 5 mM HCl: acetone, and 1 mM HCl. The tresylated cross-linked agarose beads are stored at 4 °C until used. The reactivity of Sepharose tresyl groups is very high, allowing a 75 to 100% coupling yield of thiol- or amino-containing molecules within 1 hr at pH 7.5 in the cold. Thiols and primary amino groups are the most reactive nucleophiles with sulfonate esters on gels, thiols showing the highest reactivity.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the tresyl-activated cross-linked agarose beads are washed quickly with cold 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2. Thereafter, the moist agarose beads (1 g) are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the gel is treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VII.3.5. Immobilization of activated capture oligodeoxynucleotides onto tosyl-activated beaded agarose

The preparation of tosylated cross-linked beaded agarose was generally performed as described for tresyl-activated beaded agarose, except that Sepharose CL-6B (8 g, wet) and 0.6 g tosyl chloride (p-toluene sulfonyl chloride) were used instead of 10 g Sepharose 4B and 100 µl tresyl chloride. The tosyl-activated cross-linked agarose beads are washed quickly with cold 0.25 M $NaHCO_3$ at pH 10.5. Sepharose tosyl groups require a pH of 9 to 10.5 for efficient coupling of amine-containing molecules. Thereafter, the moist agarose beads (0.7 g) are resuspended in 1 ml of 0.25 M $NaHCO_3$, pH 10.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 40 °C with gentle agitation, the gel is treated with 0.8 M mercaptoethanol, pH 10, for 15 hrs at 40 °C, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VII.3.6. Immobilization of activated capture oligodeoxynucleotides onto sulfhydryl-containing polyacrylhydrazidoagarose (PAHOS)

Sepharose beads derivatized with linear polyacrylic hydrazide (PAHOS) provide properties of Sepharose and polyacrylamide gels. The preparation of PAHOS is performed as described by by Wilchek, M. et al., (Meth. Enzyol. 104, 3, 1984). Washed Sepharose 4B (10 g) is suspended in 30 ml of freshly prepared 0.25 M sodium periodate. The suspension is slowly stirred at room temperature for 3 hrs in the dark. The oxidized Sepharose is then washed with cold and resuspended in three volumes of aqueous polyacrylhydrazide solution (0.1 - 0.5%) prepared from polymethylacrylate and hydrazine hydrate as described (Wilchek, M., and Miron, T. Meth. Enzymol. 34, 72, 1974). After slow stirring for 16 hrs in the dark at room temperature, the beads are washed extensively with 0.1 M NaCl, and then reduced with 0.3 M sodium borohydride in 0.5 M Tris-HCl, pH 8, for 3 hrs at room temperature. The reduced gel is washed with water on a sintered-glass funnel and stored at 4 °C.

Sulfhydryl groups are introduced by treating the gel with N-acetylhomocysteine thiolactone. The thiolactone (1 g) is added to a cold suspension of 10 ml of PAHOS

beads in 20 ml of 1 M $NaCO_3$. After slow stirring for 16 hrs at 4 °C, the product is washed extensively with water and 0.1 M NaCl.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the sulfhydryl-activated PAHOS beads are washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the beads are resuspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After incubation for 20 hrs at room temperature with gentle agitation, the beads are washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VII.3.7. Immobilization of activated capture oligodeoxynucleotides onto tresyl-activated silica

Porous glass spheres (10 μm diameter, 500 Å pore diamter) (LiChrosphere Si, Altex Scientific, Inc., Berkeley, CA, USA) are treated with hot chromic acid cleaning solution follwed by rinsing with 100 ml per 1 g porous glass spheres of hot 1 M HNO_3 and water. Thereafter, the cleaned porous glass spheres are suspended in a 10% aqueous solution of glycidoxypropyl trimethoxysilane, degassed with ultrasonic vibration for 10 min, and kept for 2 hrs at 90 °C, during which time the pH is maintained at 3.0 with 1 M HCl. the glass sheres are then collected on a medium-porosity glass frit, rinsed with water and dried overnight at 105 °C in vacuo.

The preparation of tresylated porous glass spheres coated with a hydrophilic layer of glycerylpropyl groups is performed as described by Nilsson, K., and Mosbach, K. (Meth. Enzymol. 104, 56, 1984). The dried porous glass spheres (2 g) are washed three times with 50 ml each of dry acetone (dried with a molecular sieve overnight). The spheres are then transferred to a dried beaker containing 2.5 ml of dry acetone and 130 μl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 90 μl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 15 min at 0 °C, the spheres are washed twice with 50 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone,

70: 30 of 5 mM HCl: acetone, and 1 mM HCl. For storage, the tresylated porous glass spheres are washed with water, 50: 50 (v/v) water: acetone, and acetone and dried.

For coupling of 5'-amine derivatized oligodeoxynucleotides, the tresylated porous glass spheres are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the spheres are treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VII.3.8. Immobilization of activated capture oligodeoxynucleotides onto aldehyde-activated polyester film

The polyester film is a polymer of glycerol and terephthalic acid (Gareware Chemical Co., India) which on partial acid hydrolysis and periodate oxidation provides aldehyde groups (Sarkar, M., and Mandal, C. J. Immunol. Meth. 83, 55, 1985). For partial hydrolysis, the film is hydrolyzed with 1 M H_2SO_4 for 6 hrs at 100 °C, washed with distilled water, and then treated with 0.1 M $NaIO_4$ for 24 hrs at 30 °C. After elimination of excess periodate by the addition of 0.1 M ethylene glycol, the film is washed with distilled water and stored at 4 oC in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5.

For coupling of 5'-hydrazide derivatized oligodeoxynucleotides, the aldehyde-activated polyester film is incubated with the 5'-hydrazide-oligodeoxynucleotide derivative to be immobilized in 50 mM M sodium phosphate, 150 M NaCl, pH 7.5. After 16 hrs at room temperature with gentle agitation, the film is washed with 50 mM M sodium phosphate, 150 mM NaCl, pH 7.5. To reduce the hydrazone bonds to more stable linkages, the film may be further incubated for 1 hr in the presence of 15 mM sodium cyanoborohydride.

VII.4. SYNTHESIS OF CARRIER MOLECULES FOR ENHANCED COVALENT AMPLICATION SYSTEMS

Oligomeric or polymeric carrier molecules containining multiple reactive residues may be utilized for at least two different purposes, i) for covalent attachment to the termini of

detector and primer oligonucleotides, and ii) for assays using additional, non-attached carrier molecules. The following examples describe the synthesis of oligonucleotide carrier molecules and polymeric dextran carriers. The oligonucleotide carrier molecules are designed for covalent attachment to the termini of detector and primer oligonucleotides. Therefore, they contain one terminal type I reactive residue capable of covalent binding to the termini of reactive residue-containing detector or primer oligonucleotides, and multiple type II reactive residues capable of covalent binding multiple detector reagents. The polymeric dextran carriers are designed to be employed as additional, non-attached carrier molecules in enhanced covalent amplification systems. Therefore, they contain one or more type I reactive residues capable of covalent binding to reactive residue-containing detector oligonucleotides or reactive residue-containing amplicons of target nucleic acids, and multiple type II reactive residues capable of covalent binding multiple detector reagents.

VII.4.1. Synthesis of oligonucleotide carrier molecules

Two different methods are applied to provide primer and detector oligonucleotides with oligonucleotide carrier molecules containing multiple reactive residues. In method A, the oligonucleotide carrier molecule is synthesized first and then covalently attached to a primer or detector oligonucleotide. In method B, fully protected phosphoramidite derivatives of reactive residue-containing nucleotides are synthesized first and then attached to a primer or detector oligonucleotide using automated standard solid-phase phosphoramidite synthesis techniques.

VII.4.1.1. Synthesis of oligonucleotide carrier molecules according to method A

This example describes the synthesis of a 25-mer oligonucleotide carrier molecule containing 12 repeats of adenine-guanine and a single cytosine residue at the 3'-terminus. The oligonucleotide is synthesized using automated standard solid-phase phosphoramidite synthesis techniques and purified by HPLC. Subsequent derivatization steps include a) bromination of the guanine and adenine residues at the C-8 position, b) coupling of 1,6-diaminohexane to the brominated bases by nucleophilic displacement, c) derivatization of the terminal cytosine residue at the C-4 position with bisulfite in the presence of cystamine, d) derivatization of the introduced amino groups with thioester

residues by reaction with SATA, and e) generation of a 3'-terminal sulfhydryl residue by reduction of the cytosine-attached cystamine residue.

a) *Bromination of the guanine and adenine residues.* The oligonucleotide (50 μg) is mixed in a microfuge tube with 50 μl of 1 M sodium bicarbonate, pH 9.6, and 490 μl of water, chilled on ice, and mixed with 10 μl of N-bromosuccinimide (Sigma-Aldrich, Deisenhofen, Germany) dissolved in water at a concentration of 1.42 mg/ml (makes an 8 mM final concentration in the reaction mixture). After reaction on ice for 10 min, the bromine activated oligonucleotide is used immediately for coupling of 1,6-diaminohexane.

b) *Coupling of 1,6-diaminohexane to the brominated bases.* An aliquot of 65 μl of 1,6-diaminohexane dihydrochloride (dissolved in water at a concentration of 100 mM) is added to the brominated oligonucleotide and reacted for 1 hr at 50°C. Thereafter, excess reactants are removed by gel filtration on Sephadex G-25 equilibrated with a bisulfite modification solution consisting of 1 M sodium bisulfite, 3 M cystamine dihydrochloride (Sigma-Aldrich, Deisenhofen, Germany), pH 6.0.

c) *Derivatization of the terminal cytosine residue with bisulfite in the presence of cystamine.* The oligonucleotide dissolved in 1 M sodium bisulfite, 3 M cystamine dihydrochloride (Sigma-Aldrich, Deisenhofen, Germany), pH 6.0, is reacted for 3 hr at 42°C. Thereafter, excess reactants are removed by gel filtration on Sephadex G-25 equilibrated with 50 mM sodium phosphate, pH 8.0.

d) *Derivatization of the introduced amino groups with SATA.* The derivatized oligonucleotide in 750 μl of 50 mM sodium phosphate, pH 8.0, is mixed with 750 μl of N-succinimidyl S-acetylthioacetate (SATA; Pierce Chemical Co., Rockford, IL, USA) dissolved in DMF at a concentration of 8 mg/ml, and reacted for 3 hr at 37°C.

e) *Generation of a 3'-terminal sulfhydryl residue.* To the SATA-derivatized oligonucleotide 1 M dithiothreitol is added to a final concentration of 100 mM and after 30 min at 37°C, the oligonucleotide carrier is purified by gel filtration on Sephadex G-25

using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, ph 7.2. The purified oligonucleotide carrier is used immediately for coupling to primer or detector oligonucleotides containing a terminal sulfhydryl-reactive residue.

VII.4.1.2. Synthesis of reactive residue-containing oligonucleotides according to method B

In this example, 2'-deoxyguanosine is derivatized at the C-8 position with a haloacetyl residue and then used to prepare a fully protected phosphoramidite derivative suitable for automated standard solid-phase phosphoramidite synthesis techniques. The identical procedure can be applied for the preparation of analogous derivatives of 2'-deoxyadenosine.

a) *Bromination of 2'-deoxyguanosine.* 2'-Deoxyguanosine (50 μg) is mixed in a microfuge tube with 50 μl of 1 M sodium bicarbonate, pH 9.6, and 490 μl of water, chilled on ice, and mixed with 10 μl of N-bromosuccinimide (Sigma-Aldrich, Deisenhofen, Germany) dissolved in water at a concentration of 1.42 mg/ml (makes an 8 mM final concentration in the reaction mixture). After reaction on ice for 10 min, the bromine activated 2'-deoxyguanosine is used immediately for coupling of 1,6-diaminohexane.

b) *Coupling of 1,6-diaminohexane to brominated 2'-deoxyguanosine.* An aliquot of 65 μl of 1,6- diaminohexane dihydrochloride (dissolved in water at a concentration of 100 mM) is added to the brominated 2'-deoxyguanosine. After reaction for 1 hr at 50°C, the 2'-deoxyguanosine derivative is purified by HPLC.

c) *Derivatization of the amine terminal spacer with N-hydroxysuccinimidyl iodoacetate.* The amine-derivatized 2'-deoxyguanosine is dissolved in 50 mM sodium phosphate, pH 8.0, at a concentration of 1 mM and mixed with a 3-fold molar excess of N-hydroxysuccinimidyl iodoacetate (Rector, E.S. et al., J. Immunol. Meth. 24, 321, 1978) dissolved in DMF at a concentration of 8 mg/ml. After reaction for 3 hr at 37°C, the iodoacetyl-derivative is purified by HPLC.

d) *Preparation of a fully protected phosphoramidite derivative*. The 5' primary hydroxyl function of the iodoacetyl-derivative of 2'-deoxyguanosine is protected by a dimethoxytitryl (DMT) or trimetoxytitryl (TMT) moiety, the 3' secondary hydroxyl function is derivatized with the reactive phosphoramidite group, and the phosphate oxygen of this group is protected by either a methoxy or β-cyanoethoxy group. All protection procedures are performed according to standard methods.

VII.4.2. Activation of dextran polymers

In the following examples, dextran polymers are activated (section VII.4.2.) and then derivatized with two different reactive residues (section VII.4.3.), with one or more type I reactive residues capable of covalent binding to reactive residue-containing detector oligonucleotides or reactive residue-containing amplicons of target nucleic acids, and multiple type II reactive residues capable of covalent binding multiple detector reagents.

Various soluble dextran polymers with an average molecular weight ranging from 10,000 to 500,000 are commercially available (e.g., Sigma-Aldrich, Deisenfofen, Germany). Preferred are dextran polymers with an average molecular weight ranging from 10,000 to 100,000.

VII.4.2.1. Synthesis of polyaldehyde derivatives of dextran

Dextran of molecular weight between 10,000 and 40,000 is dissolved in a 30 mM aqueous sodium periodate solution (6.42 g $NaIO_4$ in 500 ml deionized water) and stirred overnight at room temperature in the dark. Excess reactant is removed by dialyis against water and the purified polyaldehyde dextran is lyophilized for long-term storage.

The degree of aldehyde formation may be assessed by aldehyde-mediated reduction of Cu^{2+} to Cu^{+} which can be detected using the bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA) as described by Smith, P.K. et al., (Anal. Biochem. 150, 76, 1985). The formation of Cu^{+} is in direct proportion to the amount of aldehydes present in the polymer. BCA forms a purple-colored complex with Cu^{+} which can be measured at 562 nm.

VII.4.2.2. Synthesis of polyamine and polyhydrazide derivatives of dextran

For the preparation of polyamine derivatives of dextran, ethylene diamine (or another suitable diamine) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 3 M. To avoid pH adjustment of the highly alkaline free-base form of ethylene diamine, the hydrochloride form of ethylene diamine is utilized. For the preparation of polyhydrazide derivatives of dextran, adipic acid dihydrazide (or another suitable dihydrazide compound) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 30 mg/ml (heating under a hot water tap is necessary to dissolve the dihydrazide compound completely at this concentration) and the pH is adjusted to 7.2 with HCl. The diamine- or dihydrazide-containing solution is used to dissolve polyaldehyde dextran (prepared as described in section VII.4.2.1.) at a concentration of 25 mg/ml and each ml of the polyaldehyde dextran/diamine (or polyaldehyde dextran/dihydrazide) solution is mixed with 0.2 ml of 1 M sodium cyanoborohydride. After reaction for at least 6 hrs at room temperature, excess diamine (or dihydrazide) and reaction-by-products are removed by dialysis.

VII.4.2.3. Synthesis of polycarboxymethyl derivatives of dextran

In a fume hood, a solution consisting of 1 M chloroacetic acid in 3 M NaOH is prepared and immediately used to dissolve dextran polymer at a concentration of 40 mg/ml. After reaction for 70 min at room temperature with stirring, the reaction is stopped by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 M HCl. Excess reactants are removed by dialysis.

VII.4.2.4. Synthesis of lactone derivatives of polycarboxymethyl-dextran

The lactone derivative of polycarboxymethyl-dextran is prepared by refluxing polycarboxymethyl-dextran (section VII.4.2.3.) for 5 hrs in toluene or in other anhydrous solvents as described by Heindel, N.D. et al. (Bioconjugate Chem. 5, 98, 1994). The lactone derivative is highly reactive towards amine-containing molecules.

VII.4.2.5. Synthesis of epoxy-activated derivatives of dextran

*Bis*oxirane compounds are utilized to introduce epoxide functional groups into soluble dextran polymers as described by Böcher, M. et al. (J. Immunol. Meth. 151, 1, 1992).

Epoxide functional groups react efficiently with sulfhydryl groups at pH values ranging between 7.5 and 8.5, and with amine nucleophiles at moderate alkaline pH values (typically needing pH values of at least 9).

In a fume hood, 1,4-butanediol diglycidyl ether is mixed with an equal part of 0.6 M NaOH containing 2 mg/ml sodium borohydride. With stirring, 5 mg of dextran are added to each ml of the *bis*-epoxide solution. After reaction for 12 hrs at 25 °C, excess reactants are removed by extensive dialysis. For long-term storage, the activated dextran is lyophilized.

VII.4.3. Synthesis of polymeric dextran carriers containing two different reactive residues

VII.4.3.1. Synthesis of dextran carriers containing hydrazide and pyridyl disulfide residues

For the preparation of dextran carriers containing both hydrazide and pyridyl disulfide residues, polyhydrazide-derivatized dextran polymers (prepared as described in section VII.4.2.2.) are reacted with limited amounts of the heterobifunctional cross-linking reagent SPDP (Pierce Chemical Company, Rockford, IL, USA). The hydrazide residues may be used for covalent attachment to haloacetyl-containing detector oligonucleotides, and the pyridyl disulfide residues for covalent binding of sulfhydryl-containing detector reagents. Using this assay design, hydrazide residues and pyridyl disulfide residues should be present at a ratio of approximately 2:8.

SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution) and 100 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyhydrazide-derivatized dextran polymer at a concentration of 20 mg/ml. After reaction for 1 hour at room temperature, excess reagents are removed by dialysis. If necesssary, the degree of pyridyl disulfide derivatization can be easily adjusted by either changing the concentration of SPDP and/or varying the incubation period. The degree of pyridyl disulfide derivatization is determined by monitoring the release of pyridine-2-thione (characteristic absorbance at 343 nm; $\varepsilon = 8.08 \times 10^3\ M^{-1}$

cm^{-1}) after incubation of an aliquot for 15 min at room temperature in the presence of 20 mM dithiothreitol.

VII.4.3.2. Synthesis dextran carriers containing of thioester and pyridyl disulfide residues

For the preparation of dextran carriers containing both thioester and pyridyl disulfide residues, polyhydrazide-derivatized dextran polymers (prepared as described in section VII.4.2.2.) are reacted with the heterobifunctional cross-linking reagents SATA (Pierce Chemical Company, Rockford, IL, USA) and SPDP (Pierce Chemical Company, Rockford, IL, USA). The pyridyl disulfide residues may be used for covalent attachment to sulfhydryl-containing detector oligonucleotides, and the thioester residues (after pretreatment with neutral hydroxylamine) for covalent binding of haloacetyl-containing detector reagents. Using this assay design, pyridyl disulfide residues and thioester residues should be present at a ratio of approximately 2:8.

SATA (MW 231.2) is dissolved at a concentration of 4.6 mg/ml in DMF (20 mM) and SPDP (MW 312.4) is dissolved at a concentration of 6.2 mg/ml in DMF (20 mM). From the SATA solution 80 µl and from the SPDP solution 20 µl are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyhydrazide-derivatized dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis. If necesssary, the degree of derivatization with each reagent can be easily adjusted by changing their relative concentrations.

The degree of pyridyl disulfide derivatization is determined by monitoring the release of pyridine-2-thione (characteristic absorbance at 343 nm; $\varepsilon = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$) after incubation of an aliquot for 15 min at room temperature in the presence of 20 mM dithiothreitol. The degree of thioacetyl derivatization may be determined by measuring the amount of hydrazide residues before and after derivatization with SATA and SPDP.

VII.4.3.3. Synthesis of dextran carriers containing iodoacetyl and thioester residues

For the preparation of dextran carriers containing both thioester and iodoacetyl residues, polyhydrazide-derivatized dextran polymers (prepared as described in section

VII.4.2.2.) are reacted with the heterobifunctional cross-linking reagents SATA (Pierce Chemical Company, Rockford, IL, USA) and SIAX (Brinkley, M. Bioconjugate Chem. 3, 2, 1992). The iodoacetyl residues may be used for covalent attachment to sulfhydryl-containing detector oligonucleotides, and the thioester residues (after pretreatment with neutral hydroxylamine) for covalent binding of haloacetyl-containing detector reagents. Using this assay design, iodoacetyl and thioester residues should be present at a ratio of approximately 2:8.

SATA (MW 231.2) is dissolved at a concentration of 4.6 mg/ml in DMF (20 mM) and SIAX (MW 396) is dissolved at a concentration of 7.9 mg/ml in DMF (20 mM). From the SATA solution 80 µl and from the SIAX solution 20 µl are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyhydrazide-derivatized dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis. If necesssary, the degree of derivatization with each reagent can be easily adjusted by changing their relative concentrations.

The degree of iodoacetyl derivatization can be determined using β-mercaptoethanol for reaction with the iodoacetyl residues and Ellman's reagent for quantification of the remaining β-mercaptoethanol. The degree of thioacetyl derivatization may be determined by measuring the amount of hydrazide residues before and after derivatization with SATA and SPDP.

VII.5. PREPARATION OF ENZYMATIC DETECTOR REAGENTS

The following examples describe the preparation of enzymatic detector reagents with alkaline phosphatase activity. In section VII.5.1., alkaline phosphatase is derivatized with different reactive residues which are suitable for covalent coupling to reactive residue-containing amplicons of target nucleic acids, reactive residue-containing detector oligonucleotdes, or reactive residue-containing carrier molecules. Section VII.5.2. describes the preparation of enzymatic detector reagents containing multiple alkaline phosphatase molecules immobilized onto polymeric dextran carrier molecules.

VII.5.1. Derivatization of alkaline phosphatase with reactive residues

VII.5.1.1. Derivatization of alkaline phosphatase with pyridyl disulfide residues

Alkaline phosphatase is dissolved at a concentration of 10 mg/ml in 50 mM sodium phosphate, 3 M NaCl, pH 7.4 (or dialyzed against if the enzyme is dissolved in another buffer). To each ml alkaline phosphatase solution, 15 µl of 20 mM LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) in DMF (8.5 mg/ml) is added and mixed (for different concentrations of alkaline phosphatase in the reaction medium, the amount of LC-SPDP is adjusted proportionally). After incubation for 30 min at room temperature, the solution is subjected to gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4. At this point, the derivative is stable and may be stored.

The degree of substitution can be determined with an aliquot of the purified LC-SPDP-derivatized enzyme at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 $M^{-1}cm^{-1}$) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM.

VII.5.1.2. Derivatization of alkaline phosphatase with thioester residues

Alkaline phosphatase is dissolved at a concentration of 10 mg/ml in 50 mM sodium phosphate, 3 M NaCl, 1 mM $MgCl_2$, pH 7.4 (or dialyzed against if the enzyme is dissolved in another buffer). To each ml alkaline phosphatase solution, 15 µl of 20 mM SATA (Pierce Chemical Company, Rockford, IL, USA) dissolved in DMSO at a concentration of 4.6 mg/ml is added (for different concentrations of alkaline phosphatase in the reaction medium, the amount of SATA is adjusted proportionally). After 30 min at room temperature, the SATA-derivatized enzyme is purified by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.4. At this point, the derivative is stable and may be stored.

VII.5.1.3. Derivatization of alkaline phosphatase with sulfhydryl residues

Sulfhydryl residue-containing alkaline phosphatase is prepared from the SATA-derivatized enzyme. For deprotection of the acetylated sulfhydryl groups, each ml of

SATA-derivatized alkaline phosphasrase is mixed with 100 µl of 0.5 M hydroxylamine in 0.1 M sodium phosphate, 10 mM EDTA, pH 7.4. After 2 hrs at room temperature, the thiolated enzyme is purified by gel filtration on Sephadex G-25 equilibrated with 0.05 M sodium phosphate, 3 M NaCl, 10 mM EDTA, pH 7.4. The sulfhydryl-derivatized enzyme is used immediately for coupling (e.g., to sulfhydryl-reactive polymeric carrier molecules).

VII.5.1.4. Derivatization of alkaline phosphatase with aldehyde residues

Alkaline phosphatase is dissolved at a concentration of 10 mg/ml in 100 mM sodium bicarbonate, 3 M NaCl, pH 8.5 (or dialyzed against if the enzyme is dissolved in another buffer). To 0.4 ml of the alkaline phosphatase solution, 20 µl of 50 mM SFB (Pierce Chemical Company, Rockford, IL, USA) dissolved in acetonitrile at a concentration of 12.35 mg/ml. After 30 min at room temperature, the SFB-derivatized enzyme is purified by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4. The purified enzyme derivative is utilized immediately for coupling (e.g., to hydrazide-containing polymeric carrier molecules).

VII.5.2. Immobilization of alkaline phosphatase onto polymeric carrier molecules

VII.5.2.1. Coupling of alkaline phosphatase to polyaldehyde-dextran

In this example, non-derivatized alalkaline phosphatase is coupled to polyaldehyde derivatives of dextran. The polyaldehyde derivative of dextran (prepared as described in section VIIII.4.2.1.) is dissolved in 0.1 M sodium bicarbonate, 3 M NaCl, pH 8.5, at a concentration of 20 mg/ml. To each ml of polyaldehyde-dextran solution 10 mg alkaline phosphatase dissolved in 1.0 ml of 0.1 M sodium bicarbonate, 3 M NaCl, pH 8.5, is added. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride is added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the dextran-alkaline phosphatase conjugates are purified by gel filtration using a column of Sephacryl S-200 or S-300.

VII.5.2.2. Coupling of sulfhydryl-derivatized alkaline phosphatase to polypyridyl disulfide-dextran

For the preparation of polypyridyl disulfide-dextran carrier molecules, polyhydrazide-derivatized dextran polymers (prepared as described in section VII.4.2.2.) are reacted with excess amounts of the heterobifunctional cross-linking reagent SPDP (Pierce Chemical Company, Rockford, IL, USA). SPDP is dissolved at a concentration of 9.3 mg/ml in DMF (makes a 30 mM stock solution) and 200 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyhydrazide-derivatized dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis. The degree of pyridyl disulfide derivatization is determined by monitoring the release of pyridine-2-thione (characteristic absorbance at 343 nm; $\varepsilon = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$) after incubation of an aliquot for 15 min at room temperature in the presence of 20 mM dithiothreitol.

To each ml of polypyridyl disulfide-dextran solution 10 mg of sulfhydryl-derivatized alkaline phosphatase (prepared as described in section VII.5.1.3.) in 1.0 ml of 0.05 M sodium phosphate, 3 M NaCl, 10 mM EDTA, pH 7.4, is added. After incubation of the reaction mixture for 3 hours at room temperature, the dextran-alkaline phosphatase conjugates are purified by gel filtration using a column of Sephacryl S-200 or S-300.

VII.6. PREPARATION OF LIPOSOMAL DETECTOR REAGENTS

The examples of section VII.6. describe the preparation of non-complexed liposomal detector reagents which contain one type of surface-attached reactive residue capable of covalent coupling to reactive residue-containing amplicons of target nucleic acids, detector oligonucleotides, and polymeric carrier molecules. The reactive residues are attached to the surface of liposomal detector reagents either by incorporation of reactive-residue-derivatized lipid components (for synthesis, see section VII.6.1.), or by derivatization of lipid components with reactive residues after formation of the intact liposomal detector reagent (for synthesis, see section VII.6.4.).

VII.6.1. Activation of phosphatidyl ethanolamine with reactive residues

VII.6.1.1. Activation of phosphatidyl ethanolamine with a pyridyl disulfide residue

Phosphatidyl ethanolamine (PE) (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (LC-PDP-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The LC-PDP-PE derivative is further purified by chromatography on a column of silicic acid as described by Martin, F.J. et al., (*In*: Liposomes, A Practical Approach, pp. 163-182, IRL Press, New York, 1990). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified LC-PDP-PE by TLC as described above. Finally, the chloroform is removed from the LC-PDP-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VII.6.1.2. Activation of phosphatidyl ethanolamine with a maleimide residue

Phosphatidyl ethanolamine (PE) (100 μmol) is dissolved in 5 ml of argon-purged, anhydrous methanol containing 100 μmol of triethylamine (TEA) and maintained over an argon or nitrogen atmosphere. After the addition of 50 mg SMPB (Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel

60-F_{254} plates (Merck, Germany) developed with a 65:25:4 (by volume) mixture of chloroform: methanol: water. The activated PE derivative develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The MPB-PE derivative is further purified by chromatography on a column of silicic acid as described by Martin, F.J. et al., (Biochemistry 20, 4229, 1981). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform : methanol mixtures: 4 : 0.25, 4 : 0.5, 4 : 0.75, and 4 : 1. Fractions of 2 ml are collected and monitored for the presence of purified MPB-PE by TLC as described above. Finally, the chloroform is removed from the MPB-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VII.6.1.3. Activation of phosphatidyl ethanolamine with an iodoacetyl residue

Phosphatidylethanolamine (PE) (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SIAXX (Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (SIAXX-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform and purified by chromatography on a column of silicic acid as described by Martin, F.J. et al. (see section VII.6.1.1 above). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:

methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified IAXX-PE by TLC as described above. Finally, the chloroform is removed from the IAXX-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VII.6.1.4. Activation of phosphatidyl ethanolamine with an aldehyde group

Phosphatidyl ethanolamine (PE) (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SFPA (Molecular Probes, Eugene, OR, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (FPA-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform and purified by chromatography on a column of silicic acid as described by Martin, F.J. et al, (see section VII.6.1.1 above). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified FPA-PE by TLC as described above. Finally, the chloroform is removed from the FPA-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VII.6.1.5. Activation of phosphatidyl ethanolamine with a thioester residue

Phosphatidyl ethanolamine (PE) (15 mg; 20 μmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 μmol SATP (Molecular Probes, Eugene, OR, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere.

Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (ATP-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The ATP-PE derivative is further purified by chromatography on a column of silicic acid as described by Martin, F.J. et al. (see section VII.6.1.1 above). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified ATP-PE by TLC as described above. Finally, the chloroform is removed from the ATP-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VII.6.2. Preparation of liposomal detector reagents containing pyridyl disulfide-derivatized phosphatidylethanolamine

The follwing examples describe the preparation of non-complexed liposomal detector reagents utilizing pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) as reactive residue-containing lipid derivative. The liposomal detector reagents contain either encapsulated *p*-aminophenol for electrometric detection procedures or the highly water-soluble 5(6)-carboxyfluorescein (CF) for fluorescent detection procedures. For encapsulation, both reporter molecules are dissolved in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer in distilled water, pH 7.4, the redox mediator *p*-aminophenol at a concentration of 50 mM and 5(6)-carboxyfluorescein at a concentration of 200 mM.

VII.6.2.1. Purification of 5(6)-carboxyfluorescein (CF)

Hydrophobic impurities in preparations of fluorescent reporter molecules pose a potential problem for efficient liposomal enapsulation. Therefore, manny reporter

molecules require purification prior to encapsulation. The following examples describe the purification of CF, since several commercially available CF preparations contain both hydrophilic and hydrophobic impurities.

Method A In method A, hydrophobic impurities are removed by elution of CF with water on a hydrophobic LH20 Sephadex column (Pharmacia, Piscataway, NJ, USA) as described by Weinstein, J.N. et al., (In: Liposome Technology, vol. III (G. Gregoriadis, ed.) pp. 183-204, CRC Press, Inc., Boca Raton, FL, USA, 1984).

CF (35 g) is dissolved in 200 ml of absolute ethanol in an Erlenmeyer flask. After the addition of 2 g of 'Norit A' (activated charcoal; Aldrich), the solution is boiled for 5 min and then filtered through a Buchner funnel with Whatman No. 50 filter paper. The filtrate is collected, mixed slowly with cold water (approximately 400 ml; with magnetic stirring) until cloudiness persists, further cooled, and then placed in a freezer (-20 °C) overnight. The formed CF precipitate (fluffy, light orange powder) is placed on a Buchner funnel, washed extensively (use of suction) with iced distilled water (no ethanol smell should remain), dried (first in a Buchner funnel, then in a desiccator with $CaCl_2$), and used to prepare a 250 mM CF solution in distilled water by titrating to pH 7.4 with 1 M NaOH. An aliquot of 50 ml of this solution is passed over a 40 cm x 5 cm column of LH20 Sephadex (Pharmacia) and eluted with distilled water. Impurities across the broad CF band are analyzed by TLC on silica plates usinga mixture of $CHCl_3$: CH_3OH: H_2O (65: 25: 4) as solvent. Purified CF cn be stored in the refrigerator (protected from light) for several months with little deterioration.

Method B In method B, CF is purified by reverse-phase HPLC as described by Weinstein, J.N., see Method A. An aliquot of an aqueous solution of CF (10 mM) is passed over a C18 reverse-phase column and eluted at room temperature with a linear gradient of methanol in 0.5% acetic acid. The gradient (25 to 100% methanol) is run in 20 min at a flow rate of 2 ml/min. The elution of CF is monitored at 254 nm.

VII.6.2.2. Preparation of MLV liposomes using pyridyl disulfide-derivatized PE

MLV liposomes containing encapsulated *p*-aminophenol (or CF) are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidylcholine (PC), cholesterol, dipalmitoyl phosphatidylglycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidylethanolamine (PD-DPPE) of 8:10:1:1. Using this percentage of cholesterol, the integrity of the liposomal bilayer will be stable up to a level of organic solvent addition of about 5%.

The lipid mixture solved in organic solvent (approximatey a total of 60 μmol/ml preparation) is pipetted into a round-bottom flask and then dried under reduced pressure at or close to its transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration). Thereafter, the lipid mixture is hydrated with *p*-aminophenol dissolved at a concentration of 50 mM (or CF dissolved at a concentration of 200 mM) in 10 mM HEPES buffer, pH 7.4, by vortexing for at least 10 min in a water bath at or above the transition temperature of the lipid mixture. Next, the lipid mixture is mechanically shaken in the water bath for about 30 min and after another 30 min at room temperature (without skaking), the liposome mixture is filtered through 0.4 μm nucleopore filter under nitrogen pressure. Sometimes it is necessary to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. The MLVs are dialyzed against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.6.2.3. Preparation of SUV liposomes using pyridyl disulfide-derivatized PE

SUVs are made from the MLV. After the first step in the MLV procedure (hydration by vortexing), the lipid mixture is transferred to a sonicating flask, equipped with a long neck and cap with in and out spouts for nitrogen. Nitrogen is flushed through the flask, then a light stream of nitrogen is left going into the tube and the outlet is closed. The flask is placed into a sonicator (e.g., Laboratory Supplies Company, Hicksville, NY, USA) at or above the highest transition temperature of any one lipid in the mixture. Sonication is performed for 30 to 60 min until the mixture appears opalescent.

Thereafter, the SUVs are allowed to equilibrate for approximately 30 min at room temperature before uncaptured reporter molecules are removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.6.2.4. Preparation of LUV liposomes using pyridyl disulfide-derivatized PE

For the preparation of LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C., and Paphadjopoulos, D. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting, on a molar ratio basis, of dipalmitoyl phosphatidylcholine (PC), cholesterol, dipalmitoyl phosphatidylglycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) of 8:10:1:1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids are redissolved in isopropyl ether, freshly redistilled from sodium bisulfite, to a concentration of approximately 20 μmol/ml ether and transferred to a 50 ml screw-cap Erlenmeyer flask. Thereafter, the aqueous phase consisting of 50 mM *p*-aminophenol (or 200 mM CF) in 10 mM HEPES buffer in distilled water, pH 7.4, is added directly to the lipid solution in a ratio of 1:3 with ether. The flask is sealed with nitrogen, contents are mixed very well, and the mixture is sonicated at room temperature for at least 5 min until the mixture looks homogeneous. The organic phase is then removed by rotary evaporation under reduced pressure initially at about 450 mm Hg for small preparations and 550 mm Hg for larger preparations. When gel forms, vacuum is increased gradually to a maximum of 700 to 750 mm Hg. Foaming during this process can be eliminiated by quick flushing of nitrogen into the flask. The temperature is also increased gradually from room temperature to about 37 °C towards the end of evaporation. At the end of the process, the residue is slightly less in volume than the original aqueous phase (at this stage, no odor of isopropyl ether should be detectable). Thereafter, the SUVs are allowed to equilibrate for approximately 30 to 60 min at room temperature, then extruded through 0.4 μm nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. Uncaptured reporter molecules are removed by dialysis against 10 mM phosphate-buffered saline,

pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.6.3. Preparation of temperature-sensitive liposomal detector reagents containing phosphatidylglycerol

Since the goal of the following examples is to produce liposomal detector reagents that undergo a sharp increase in release of encapsulated redox mediators at T_m, it is necessary to minimize lipid-soluble contaminating substances. This condition is usually met by using synthetic lipids of greater than 99% fatty acid purity. In the described examples, the synthetic lipids dipalmitoyl phosphatidylcholine (DPPC) (T_m = 41 °C), dipalmitoyl phosphatidylglycerol (DPPG) (T_m = 41 °C), and distearoyl phosphatidylcholine (DSPC) (T_m = 54 °C) are utilized. Incorporation of DPPG into the liposomal bilayer provides periodate-oxidizable vicinal hydroxyl groups which can be utilized to generate surface-attached aldehyde functions for covalent coupling of affinity components. The temperature-sensitive liposomal detector reagents contain either encapsulated *p*-aminophenol for electrometric detection procedures or the highly water-soluble 5(6)-carboxyfluorescein (CF) for fluorescent detection procedures. For encapsulation, both reporter molecules are dissolved in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer in distilled water, pH 7.4, the redox mediator *p*-aminophenol at a concentration of 50 mM and 5(6)-carboxyfluorescein at a concentration of 200 mM.

VII.6.3.1. Preparation of temperature-sensitive SUV liposomes

For the preparation of temperature-sensitive SUV liposomes, the lipid formulation consists of DPPC, DPPG, and DSPC at a molar ratio of 6.5: 0.5: 3.0. The DSPC is added to counterbalance the lowering of T_m due to the small radius of curvature of SUV. The lipids are dried from benzene onto a glass vial under a stream of argon and lyophilized overnight. A 5 ml aliquot of the 50 mM *p*-aminophenol (or 200 mM CF) in 10 mM HEPES, pH 7.4, is warmed in a water bath to 50 °C and added to the vial containing approximately a total of 40 mg lipids, also at 50 °C. At the same temperature, the suspension is hydrated with repeated votex-mixing for about 15 min.

Thereafter, the suspension is sonicated under argon to form SUV at 50 °C. After clarification of the suspension (usually within 5 min of sonication), the suspension is maintained above T_m for 15 min and centrifuged briefly at low speed to remove insoluble material. Finally, non-encapsulated reporter molecules are removed by size exclusion chromatography on Sepharose 4B (Pharmacia) equilibrated in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VII.6.3.2. Preparation of temperature-sensitive LUV liposomes

For the preparation of temperature-sensitive LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C., et al. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids (approximately a total of 125 mg) are redissolved in an organic phase consisting of 4 ml of chloroform and 8 ml of isopropyl ether, freshly washed with 10% sodium bisulfite. The mixture is transferred to a 50 ml screw-cap Erlenmeyer flask. The aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 50 mM *p*-aminophenol (or 200 mM CF). The organic phase (12 ml) and the aqueous phase (4 ml) are warmed to 50 °C and combined. The screw-cap Erlenmeyer flask is then filled with nitrogen gas and sealed with Teflon tape. The organic / aqueous mixture is placed in a cylindrical bath-type sonicator (e.g., Laboratory Supplies Company, Hicksville, N.Y., USA) filled with water at 45 to 50 °C, and sonicated for 5 min to form a milky, white, homogeneous emulsion. The emulsion is then transferred to a 125 ml tear-drop-shaped rotary evaporation flask. The water around the flask is kept at 50 °C. Upon lowering the pressure, the organic phase is drawn off. During this process the emulsion foams exuberantly and requires careful venting to adjust the pressure. After a period of 10 to 20 min, it is useful to add approximately 2 ml of additional HEPES buffer to replace the amount that is lost during the evaporation process. The procedure is finished when no foaming occurs at a pressure of approximately 150 mm Hg. The newly formed liposomes are allowed to anneal at 50 °C in a water bath for 30 min or longer. LUV affinity liposomes of well-defined size are formed by extrusion of this suspension through polycarbonate membranes above T_m. The liposomes are then rapidly cooled to room temperature in

an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated reporter molecules.

VII.6.3.3. Preparation of temperature-sensitive MLV liposomes

For the preparation of temperature-sensitive MLV liposomes, a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids (approximately a total of 125 mg) solved in organic solvent, the solvent is evaporated to dryness on the wall of a 100 ml round-bottom flask. The aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 50 mM *p*-aminophenol (or 200 mM CF). After heating of the aqueous phase to 50 °C, 4 ml are transferred to the flask containing the dried lipids, taking care to keep the flask above the T_m of the mixture. The flask is filled with nitrogen gas, closed with a glass stopper, and sealed with Teflon tape. Thereafter, the lipids are hydrated by repeated cycles of vortex-mixing for 15 sec followed by 1.5 min of incubation in a 50 °C water bath. The suspension is cycled 20 times in this manner and then allowed to anneal 30 min or longer in the 50 °C water bath. The liposomes are then rapidly cooled to room temperature in an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated reporter molecules.

VII.6.3.4. Derivatization of temperature-sensitive liposomal detector reagents with surface-attached aldehyde functions

Sodium periodate is dissolved in water to a concentration of 0.6 M (128 mg of sodium periodate/ ml of H_2O) and 200 µl of this stock solution is added with stirring to each milliliter of DPPG-liposomes (5 mg/ml) suspended in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. After incubation at room temperature for 30 min in the dark, the oxidized liposomes are dialyzed against 20 mM sodium borate, 0.15 M NaCl, pH 8.4, to remove unreacted periodate. Alternatively, unreacted periodate can be removed by gel filtration using a column of Sephadex G-50. The periodate-oxidized liposomes may be used immediately or stored in a lyophilized state in the presence of sorbitol as described by Friede, M. et al., (Anal. Biochem. 211, 117, 1993).

VII.6.4. Derivatization of intact liposomal detector reagents with pyridyl disulfide residues

First, LUV, SUV, and MLV liposomes containing encapsulated reporter molecules are prepared from a lipid mixture consisting, on a molar ratio basis, of PC, cholesterol, PG, and PE of 8:10:1:1 as described in section VII.6.1. Other lipid recipes may be used as long as they contain about this percentage of PE. In addition, if this level of cholesterol is maintained in the liposome, then the integrity of the bilayer will be stable up to a level of organic solvent addition of about 5%. This factor is important for adding an aliquot of the cross-linker to the liposome suspension as a concentrated stock dilution in an organic solvent. Any method of liposome formation may be used.

Derivatization of PE-liposomes with pyridyl disulfide residues can be performed with SPDP, LC-SPDP, or sulfo-LC-SPDP (all Pierce Chemical Company, Rockford, IL, USA). SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). Alternatively, LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (also makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that prevents the reagent from penetrating lipid bilayers. Thus, only the outer surfaces of the liposomes are activated using sulfo-LC-SPDP. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ml water.

To each milliliter of the liposome suspension to be modified, 25 - 50 µl of the stock solution of either SPDP or LC-SPDP in DMF is added. If sulfo-LC-SPDP is used, 50 - 100 µl of the stock solution in water is added to each milliliter of the liposome suspension. The reaction mixture is vortexed and reacted for 30 min at room temperature. Longer reaction times, even overnight, have no adverse effects. Finally, the modified liposomes are purified from reaction by-products by dialysis or gel filtration using Sephadex G-50. The derivatized liposomes may be used immediately for subsequent coupling reactions or stored in a lyophilized state in the presence of sorbitol as described by Friede, M. et al.,(Anal. Biochem. 211, 117, 1993).

VII.7. PREPARATION OF COVALENTLY LINKED COMPLEXES OF LIPOSOMAL DETECTOR REAGENTS

The examples of section VII.7. describe the preparation of covalently linked complexes of liposomal detector reagents utilizing two types of liposomal detector reagents with different surface-attached reactive residues. Type I liposomal detector reagents contain surface-attached pyridyl disulfide residues capable of covalent coupling to sulfhydryl-derivatized detector oligonucleotides or sulfhydryl-derivatized polymeric carrier molecules. Type II liposomal detector reagent contain surface-attached thioester residues which are capable, after treatment with neutral hydroxylamine, of forming covalent linkages with the pyridyl disulfide residues of liposomal detector reagent type I. Complexes of liposomal detector reagents are formed from MLV liposomes, SUV liposomes, or LUV liposomes.

VII.7.1. Preparation of liposomal detector reagents suitable for complexation

Type I liposomal detector reagents containing pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) and type II liposomal detector reagents containing thioester-derivatized dipalmitoyl phosphatidyl ethanolamine (ATP-DPPE) are prepared as described in section VII.6.2. The liposomal detector reagents contain either encapsulated *p*-aminophenol for electrometric detection procedures or the highly water-soluble 5(6)-carboxyfluorescein (CF) for fluorescent detection procedures. For encapsulation, both reporter molecules are dissolved in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer in distilled water, pH 7.4, the redox mediator *p*-aminophenol at a concentration of 50 mM and 5(6)-carboxyfluorescein at a concentration of 200 mM.

VII.7.1.1. Preparation of MLV liposomes

Pyridyl disulfide-derivatized MLV liposomes containing encapsulated *p*-aminophenol (or CF) are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-LC-DPPE;

prepared as described in section VII.6.1.1.) of 8:10:1:1. Thioester-derivatized MLV liposomes containing encapsulated *p*-aminophenol (or CF) are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and thioester-derivatized dipalmitoyl phosphatidyl ethanolamine (ATP-DPPE; prepared as described in section VII.6.1.5.) of 8:10:1:1. The lipid mixture solved in organic solvent (approximatey a total of 60 µmol/ml preparation) is pipetted into a round-bottom flask and then dried under reduced pressure at or close to its transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration). Thereafter, the lipid mixture is hydrated with *p*-aminophenol dissolved at a concentration of 50 mM (or CF dissolved at a concentration of 200 mM) in 10 mM HEPES buffer, pH 7.4, by vortexing for at least 10 min in a water bath at or above the transition temperature of the lipid mixture. Next, the lipid mixture is mechanically shaken in the water bath for about 30 min and after another 30 min at room temperature (without skaking), the liposome mixture is filtered through 0.4 µm nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 µm. The MLVs are dialyzed against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.7.1.2. Preparation of SUV liposomes

SUVs are made from the MLV. After the first step in the MLV procedure (hydration by vortexing), the lipid mixture is transferred to a sonicating flask, equipped with a long neck and cap with in and out spouts for nitrogen. Nitrogen is flushed through the flask, then a light stream of nitrogen is left going into the tube and the outlet is closed. The flask is placed into a sonicator (e.g., Laboratory Supplies Company, Hicksville, NY, USA) at or above the highest transition temperature of any one lipid in the mixture. Sonication is performed for 30 to 60 min until the mixture appears opalescent. Thereafter, the SUVs are allowed to equilibrate for approximately 30 min at room temperature before uncaptured reporter molecules are removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1

hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.7.1.3. Preparation of LUV liposomes

For the preparation of pyridyl disulfide-derivatized LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C. et al. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-LC-DPPE; prepared as described in section VII.6.1.1.) of 8:10:1:1 is used. For the preparation of thioester-derivatized LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C., and Paphadjopoulos, D. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and thioester-derivatized dipalmitoyl phosphatidyl ethanolamine (ATP-DPPE; prepared as described in section VIII.6.1.5.) of 8:10:1:1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids are redissolved in isopropyl ether, freshly redistilled from sodium bisulfite, to a concentration of approximately 20 μmol/ml ether and transferred to a 50 ml screw-cap Erlenmeyer flask. Thereafter, the aqueous phase consisting of 50 mM *p*-aminophenol (or 200 mM CF) in 10 mM HEPES buffer in distilled water, pH 7.4, is added directly to the lipid solution in a ratio of 1:3 with ether. The flask is sealed with nitrogen, contents are mixed very well, and the mixture is sonicated at room temperature for at least 5 min until the mixture looks homogeneous. The organic phase is then removed by rotary evaporation under reduced pressure initially at about 450 mm Hg for small preparations and 550 mm Hg for larger preparations. When gel forms, vacuum is increased gradually to a maximum of 700 to 750 mm Hg. Foaming during this process can be eliminiated by quick flushing of nitrogen into the flask. The temperature is also increased gradually from room temperature to about 37 °C towards the end of evaporation. At the end of the process, the residue is slightly less in volume than the original aqueous phase (at this stage, no odor of isopropyl ether should be detectable). Thereafter, the SUVs are allowed to equilibrate for approximately 30 to 60 min at room

temperature, then extruded through 0.4 μm nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. Uncaptured reporter molecules are removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VII.7.2. Preparation of covalently complexed liposomal detector reagents

Complexes of liposomal detector reagents are formed from either MLV liposomes, SUV liposomes, or LUV liposomes. Thioester-derivatized liposomes (MLV, SUV, or LUV) are deprotected by treatment with 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, for 2 hours at 37 °C. The sulfhydryl-containing liposomes are used immediately for reaction with pyridyl disulfide-derivatized liposomes (MLV, SUV, or LUV). Pyridyl disulfide-derivatized liposomes and sulfhydryl-derivatized liposomes are mixed at a ratio of 2:1. The degree of complex formation is controlled by the addition of iodoacetate. Finally, the covalently complexed liposomal detector reagents are purified by size exclusion chromatography on Sepharose 4B (Pharmacia) equilibrated in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VII.8 BASIC ASSAY PROCEDURE A

Detection of Mycobacterium tuberculosis DNA by covalent coupling of reporter molecules-containing liposomes to detector oligonucleotides

VII.8.1. Assay principle

Single-stranded (ss) oligonucleotides complementary to the 3'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA are covalently immobilized onto silica beads to serve as capture oligonucleotides. Captured *Mycobacterium tuberculosis* DNA is detected with ss oligonucleotides carrying a reactive residue at the 5'-terminus (detector oligonucleotides). The detector oligonucleotides are complementary to the 5'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA. Reactive residues of bound detector oligonucleotide are utilized for covalent binding of p-aminophenol-

containing liposomes carrying complementary reactive residues on the surface. Bound liposomes are lysed by the addition of detergent and released p-aminophenol (PAP) is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VII.8.2. Instrumentation

The analytical system applied for the detection of *Mycobacterium tuberculosis* DNA includes a micromachined flow-through assembly, a multipotentiostat, pumps, valves, and a computer (PC type). The flow is accomplished using a peristaltic pump combined with a 6-way selector valve. The micromachined flow-through assembly consists of three main parts: a hybridization chamber, a flow chamber designed as a channel structure, and the interdigitated electrode array (IDA). The hybridization chamber (volume: 100 □l) contains silica beads with covalently immobilized capture oligonucleotides and is connected via a two-way selector valve to a flow channel fabricated by double-sided anisotropic etching in silicon wafers. The lateral dimensions of the flow channel corresponds to the active electrode area (1mm x 3mm) and the channel hight is set to 200 □m. The inlet and outlet tubes are pasted in an acrylic holder and arranged on top of the electrode. The microelectrode arrays consist of platinum electrodes fabricated on thermal oxidized silicon wafers by photolithography and the lift-off technique.One chip contains four independent interdigitated electrode pairs and has an area of 8 mm x 8 mm. One of the 70 fingers of each electrode is 1.5 □m wide. Two adjacent fingers are 0.8 □m spaced.

VII.8.3. Assay components

VII.8.3.1. Oligonucleotides complementary to short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

a) ss Capture oligonucleotide (18-mer): The oligonucleotide is complementary to the 3'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 3'-terminus i) a 2-base thiophosphate thymine-Ts tag for immobilization to tresyl-activated silica beads and ii) a single T-base separating the tag from the sensing oligonucleotide sequence.

3'-TsTsT CAG CAG TCT GGG TTT TGG-5'

b) A short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

5'-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3'

c) ss detector oligonucleotide (18-mer): The detector oligonucleotide is complementary to the 5'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 5'-terminus an acetylthioacetyl residue for covalent coupling of pyridyl disulfide-derivatized liposomes containing p-aminophenol.

3'-GGC TCT CCC CTG CCT TTG-5'-acetylthioacetate

Derivatization of the 18-mer oligonucleotide at the 5'-terminus with an acetylthioacetate residue is performed in two steps as described in VII.1.1. and VII.1.4.

VII.8.3.2. Immobilized capture oligonucleotides. The thiophosphate thymine-containing capture oligonucleotides are immobilized onto tresyl-activated silica beads as described in VII.3.7.

VII.8.3.3. Pyridyl disulfide-derivatized liposomes containing p-aminophenol. Pyridyl disulfide-derivatized affinity liposomes are prepared by the injection method (Biochim. Biophys. Acta 298, 1015, 1973) from a lipid mixture of dimyristoylphosphatidylcholine, cholesterol, dicetylphosphate at a molar ratio of 5:4:1, and phosphatidylethanolamine derivatized with N-succinimidyl 6-(3-(2-pyridyldithio)propionamido)hexanoate (LC-SPDP) at a concentration of 0.5 mol% of total lipid. PE-LC-PDP is prepared as described in VII.6.1. To prepare liposomes, 2 µmol of stock lipid mixture in chloroform is evaporated under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid is resolubilized in 50 µl of dry isopropanol and injected with a syringe into 1 ml of 10 mM HEPES, pH7.4, containing 50 mM p-aminophenol, which is being mixed by vortex. Liposomes of uniform size are formed spontaneously by this method. After removal of unencapsulated p-aminophenol by gel filtration, liposomes are suspended in 20 mM sodium phosphate, 0.1 M NaCl, 2.5 mM EDTA, pH 7.5.

VII.8.4. Assay procedure

First, the analytical system is washed with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Therafter, the valve connecting the hybridization chamber with the flow chamber is closed and the sample (50 µl) containing the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA is applied to the hybridization chamber. After an incubation of 1 hr at room temperature, the hybridization chamber is washed with several volumes of of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0, captured *Mycobacterium tuberculosis* DNA is detected by the addition of 50 µl of 50 mM hydroxylamine hydrochloride, 0,1 M NaCl, 2.5 mM EDTA, pH 7.5, containing the detector oligonucleotide (approximately 500 ng oligonucleotide ml^{-1}). After an incubation for an additional 1 hr at room temperature, the hybridization chamber is washed again with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, 2.5 mM EDTA, pH 7.5, and 50 µl of pyridyl disulfide--derivatzed liposomes (suspended in of 20 mM sodium phosphate, 0.1 M NaCl, 2.5 mM EDTA, pH 7.5) is added. After 15 min at room temperature, the valve connecting the hybridization chamber with the flow chamber is opened and covalently copuled liposomes are lysed by the addition of 100 µl of 10 mM sodium phosphate, pH 7.0, containing 0.01% Triton X-100. Released p-aminophenol (PAP) is detected by redox recycling. PAP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current, and the quinoneimine is reduced to PAP at the cathode (-50 mV).

VII.8.5. Detection sensitivity

Using redox recyling for the detection of PAP, the detection limit is in the range of 50 nM corresponding to 2.5 pmol per 50 µl. Since 10^4 - 10^5 p-aminophenol molecules are released from a single covalently coupled liposome, the described assay configuration allows the detection of low nanogram quantities per ml of the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA (1 ng corresponds to 85 fmol).

VII.9. BASIC ASSAY PROCEDURE B

Detection of Mycobacterium tuberculosis DNA by covalent coupling of alkaline phosphatase to detector oligonucleotides

VII.9.1. Assay principle

Single-stranded (ss) oligonucleotides complementary to the 3'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA are covalently immobilized onto silica beads to serve as capture oligonucleotides. Captured *Mycobacterium tuberculosis* DNA is detected with ss oligonucleotides carrying an amine-reactive residue at the 5'-terminus (detector oligonucleotides). The detector oligonucleotides are complementary to the 5'-terminus of short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA. The amine-reactive rersidues of bound detector oligonucleotide are utilized for covalent binding of non-modified alkaline phosphatase molecules. p-Aminophenol (PAP) generated from p-aminophenylphosphate (PAPP) by covalently coupled alkaline phosphatase is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VII.9.2. Instrumentation

The analytical system applied for the detection of *Mycobacterium tuberculosis* DNA using the basic assay procedure B is the same as described in VII.8.2.

VII.9.3. Assay components

VII.9.3.1. Oligogonucleotides complementary to short fragments from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

a) ss Capture oligonucleotide (18-mer): The oligonucleotide is complementary to the 3'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 3'-terminus i) a 2-base thiophosphate thymine-Ts tag for immobilization to tresyl-activated silica beads and ii) a single T-base separating the tag from the sensing oligonucleotide sequence.

3'-TsTsT CAG CAG TCT GGG TTT TGG-5'

b) A short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA.

5'-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3'

c) ss Detector oligonucleotide (18-mer): The oligonucleotide is complementary to the 5'-terminus of the short fragment from the direct-repeat region of the *Mycobacterium tuberculosis* DNA and includes at the 5'-terminus an iodoactyl residue.

3'-GGC TCT CCC CTG CCT TTG-5'-iodoacetyl residue

Derivatization of the 18-mer oligonucleotide at the 5'-terminus with an iodoacetyl residue is performed in two steps. First, an amine terminal spacer molecule is attached to the 5'-phosphate of the 18-mer oligonucleotide as described in VII.1.1. Thereafter, the amine-derivatized oligonucleotide dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, is mixed with 250 µl of SIAX (Bioconjugate Chem. 3, 2, 1992) dissolved at a concentration of 7.9 mg/ml in DMF (20 mM). After reaction for 3 hrs at 37 °C, excess reagents are removed from the modified oligonucleotide by gel filtration.

VII.9.3.2. Immobilized capture oligonucleotides. The thiophosphate thymine-containing capture oligonucleotides are immobilized onto tresyl-activated silica beads as described in VII.3.7.

VII.9.3.3. Alkaline phosphatase. Highly purified calf intestine alkaline phosphatase (EC 3.1.3.1.; MW 140 kDa; catalytic rate constant: 3500 s^{-1}) was used.

VII.9.4. Assay procedure

First, the analytical system is washed with several volumes of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0. Therafter, the valve connecting the hybridization chamber with the flow chamber is closed and the sample (50 µl) containing the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA is applied to the hybridization chamber. After an incubation of 1 hr at room temperature, the hybridization chamber is washed with several volumes of of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0, captured *Mycobacterium tuberculosis* DNA is detected by the addition of 50 µl of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.0, containing the iodoacetyl-derivatized detector oligonucleotide (approximately 500 ng oligonucleotide ml^{-1}). After an incubation for an additional 1 hr at room temperature, the hybridization chamber is washed again with several volumes of 50 mM sodium carbonate, 01 M NaCl, pH 9.5, followed by the addition of 50 µl of highly purified, non-

derivatized alkaline phosphatase in 50 mM sodium carbonate, 01 M NaCl, pH 9.5. After 30 min at room temperature, the hybridization chamber is washed again with several volumes of 50 mM sodium carbonate, 01 M NaCl, pH 9.5, follwed by the addition of 50 µl of 100 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM diethanolamine, pH 9.6, containing 5 mM p-aminophenylphosphate (PAPP). After 15 min at room temperature, the valve connecting the hybridization chamber with the flow chamber is opened and enzymatically generated p-aminophenol (PAP) is detected by redox recycling. PAP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current, and the quinoneimine is reduced to PAP at the cathode (-50 mV).

VII.9.5. Detection sensitivity

The described assay configuration allows the detection of low nanogram quantities per ml of the short fragment (36-mer) from the direct-repeat region of the *Mycobacterium tuberculosis* DNA (1 ng corresponds to 85 fmol). .As compared to the detection sensitivity of the basic assay procedure A (VII.9.5), covalent coupling of alkaline phosphatase to hybridized detector oligonucleotides increases the sensitivity by approximately one order of magnitude as a result of the high catalytic rate constant of alkaline phosphatase. Since no prior derivatization of the enzyme is required, the enzymatic activity is maximally retained by this methodology.

* * *

CLAIMS:

1. System for the detection and quantification of a nucleic acid in a liquid sample, comprising:
(a) a solid support having immobilized thereon capture molecules capable of binding to the nucleic acid to be detected or to amplicons thereof,
(b) primer oligonucleotides for polymerase-mediated amplification of the nucleic acid to be detected, containing at least one of a first reactive residue R1, and
(c) a detector reagent comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residue R1 of the said primer oligonucleotides or to third reactive residues R3 of molecules capable of being bound to the said first reactive residue of the said primer oligonucleotide via a covalent linkage.

2. System according to claim 1, wherein the reactive residues R1 are selected from the group consisting of amines, hydrazides, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

3. System according to claim 1, wherein the reactive residues R2 are selected from the group consisting of amines, hydrazides, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, aldehydes, ketone residues, maleimides, aryl halides, alkyl halides, haloacetyl residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

4. System according to claim 1, wherein the detector reagent of (c) comprises a detector unit selected from the group consisting of enzymes capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules, and liposomes containing colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules or molecules mediating the generation of such molecules.

5. System according to claim 1, wherein the primer oligonucleotides of (b) contain an oligomeric or polymeric carrier molecule covalently attached thereto, the oligomeric or polymeric carrier molecule carrying more than one of the said first reactive residues R1.

6. System according to claim 5, wherein the oligomeric or polymeric carrier molecule is selected from hydrophilic oligomeric or polymeric derivatives of nucleotides, saccharides, amino acids, and vinyl alcohols.

7. System according to claim 1, wherein the detector reagent of (c) comprises a polymeric carrier molecule to which more than one detector unit is attached, the detector unit preferably being selected from enzymes immobilized to the polymeric carrier molecules and capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules.

8. System according to claim 7, wherein the polymeric carrier molecule comprised in the detector reagent is selected from the group consisting of hydrophilic synthetic or hydrophilic natural polymeric derivatives of nucleotides, saccharides, amino acids, vinyl alcohols, vinylpyrrolidinones, acrylic acids, urethanes, phosphazenes, and copolymer preparations of such polymeric derivatives.

9. System according to claim 7, wherein the detector units attached to the polymeric carrier molecule are enzymes immobilized on the said polymeric carrier molecule which are underivatized or are derivatized with second reactive residues R2 preferably selected from the group consisting of pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues vinylsulfone residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

10. System according to claim 1, additionally comprising:

(d) polymeric carrier molecules containing

(i) at least one reactive residue R3 capable of forming a covalent likage to the said first reactive residues R1 of the said primer oligonucleotides of (b) and

(ii) more than one reactive residue R3 capable of forming a covalent linkage to the said second reactive residues R2 of the said detector reagents.

11. System according to claim 10, wherein the reactive residue(s) R3 according to (i) and the reactive residues R3 according to (ii) are identical or different and each are preferably selected from the group consisting of amines, hydrazides, aldehydes, ketone residues, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, maleimides, aryl halides, alkyl halides, haloacetyl residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

12. System according to claim 10, wherein the oligomeric or polymeric carrier molecules are selected from hydrophilic synthetic or hydrophilic natural polymeric derivatives of nucleotides, saccharides, amino acids, vinyl alcohols, vinylpyrrolidinones, acrylic acids, urethanes, phosphazenes, and copolymer preparations of such polymeric derivatives.

13. System according to claim 1, wherein the detector reagent of (c) comprises a complex of molecules each containing at least one detector unit, at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said primer oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotide via a covalent linkage, and at least one fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2, wherein reactive residues R2 and R4 of different molecules are covalently attached to each other.

14. System according to claim 1, wherein the detector reagent of (c) comprises a complex of two different types (I and II) of molecules, type I containing at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said primer oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotides via a covalent linkage, and type II containing at least one detector unit and at least two fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2 contained in type I molecules.

15. System according to claim 14, wherein type I molecules contain an enzymatic detector unit and type 2 molecules contain a liposomal detector unit.

16. System according to claim 1, wherein the detector reagents are complexes of detector molecules each containing at least one detector unit and being crosslinked to each other by homobifunctional bridging agents via reactive residues R2 on the said detector molecules and the functional groups on the bridging agents.

17. System according to claim 16, wherein the detector reagents contain enzymatic detector units and/or liposomal detector units.

18. Method for the detection or quantification of a nucleic acid in a liquid sample, comprising the following steps:
    (a) adding primer oligonucleotides for polymerase-mediated amplification of the nucleic acid to be detected each oligonucleotide containing at least one of a first reactive residue R1 to the said sample under conditions which allow amplification of the said nucleic acid, resulting in nucleic acid amplicons carrying the said first reactive residue R1,
    (b) providing a solid support having immobilized thereon capture molecules capable of binding to the nucleic acid to be detected or to amplicons thereof,
    (c) contacting said capture molecules with the liquid sample containing nucleic acid amplicons amplified according to (a),
    (d) adding a detector reagent, comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the first reactive residue R1 of nucleic acid molecules amplified according to (a) or to third reactive residues R3 of molecules capable of being bound to the first reactive residue R1 of the said nucleic acid molecules amplified according to (a) via a covalent linkage,
    (e) removing unbound detector reagent, and
    (f) detecting or quantifying the presence of reporter molecules provided or generated by the said detector units.

19. Method according to claim 18, wherein the detector reagent used according to (d) is selected from the group consisting of (i) complexes of molecules each containing at least one detector unit, at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said primer oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotides via a covalent linkage, and at least one fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2, wherein reactive residues R2 and R4 of different molecules are covalently attached to each other, (ii) polymeric carrier molecules to each of which more than one detector unit is attached, the detector unit preferably being

selected from enzymes immobilized to the polymeric carrier molecules and capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules, and (iii) complexes of two different types (I and II) of molecules, type I containing at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said primer oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotides via a covalent linkage, and type II containing at least one detector unit and at least two fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2 contained in type I molecules.

20. Method according to claim 18, wherein said primer oligonucleotides used according to step (a) contain a covalently attached oligomeric or polymeric carrier molecule with multiple reactive residues R1 capable of covalently binding more than one detector reagent.

21. Method according to claim 18, additionally comprising

(c') adding polymeric carrier molecules containing (i) at least one reactive residue R3 capable of forming a covalent likage to the said first reactive residues R1 of the said primer oligonucleotides of (b) and (ii) more than one reactive residue R3 capable of forming a covalent linkage to the said second reactive residues R2 of the said detector reagents

after the liquid sample containing nucleic acid amplicons amplified according to (a) has been contacted with the said capture molecules and before the said detector reagent has been added.

22. System for the detection or quantification of a nucleic acid in a liquid sample, comprising:
    (a) a solid support having immobilized thereon capture molecules capable of binding to a first part but not to a second part of the nucleic acid to be detected of amplicons thereof,
    (b) detector oligonucleotides having a sequence capable of binding to the said second part of the nucleic acid to be detected or of amplicons thereof and containing at least one of a first reactive residue R1, and
    (c) a detector reagent, comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive R1 residues of the said detector oligonucleotides or to third reactive residues R3 of molecules capable of being bound to the said first reactive residue of the said detector oligonucleotides via a covalent linkage.

23. System according to claim 22, wherein the reactive residues R1 are selected from the group consisting of amines, hydrazides, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

24. System according to claim 22, wherein the reactive residues R2 are selected from the group consisting of amines, hydrazides, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, aldehydes, ketone residues, maleimides, aryl halides, alkyl halides, haloacetyl residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

25. System according to claim 22, wherein the detector reagent of (c) comprises a detector unit selected from the group consisting of enzymes capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules, and liposomes containing colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules or molecules mediating the generation of such molecules.

26. System according to claim 22, wherein the detector oligonucleotides of (b) contain an oligomeric or polymeric carrier molecule covalently attached thereto, the oligomeric or polymeric carrier molecule carrying more than one of the said first reactive residues R1.

27. System according to claim 26, wherein the oligomeric or polymeric carrier molecule is selected from hydrophilic oligomeric or polymeric derivatives of nucleotides, saccharides, amino acids, and vinyl alcohols.

28. System according to claim 22, wherein the detector reagent of (c) comprises a polymeric carrier molecule to which more than one detector unit is attached, the detector unit preferably being selected from enzymes immobilized to the polymeric carrier molecules and capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules.

29. System according to claim 28, wherein the polymeric carrier molecule comprised in the detector reagent is selected from the group consisting of hydrophilic synthetic or hydrophilic natural polymeric derivatives of nucleotides, saccharides, amino acids, vinyl alcohols, vinylpyrrolidinones, acrylic acids, urethanes, phosphazenes, and copolymer preparations of such polymeric derivatives.

30. System according to claim 28, wherein the detector units attached to the polymeric carrier molecule are enzymes immobilized on the said polymeric carrier molecule which are underivatized or are derivatized with second reactive residues R2 preferably selected from the group consisting of pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues vinylsulfone residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

31. System according to claim 22, additionally comprising:
    (d) polymeric carrier molecules containing
        (i) at least one reactive residue R3 capable of forming a covalent likage to the said first reactive residues R1 of the said detector oligonucleotides of (b) and
        (ii) more than one reactive residue R3 capable of forming a covalent linkage to the said second reactive residues R2 of the said detector reagents.

32. System according to claim 31, wherein the reactive residue(s) R3 according to (i) and the reactive residues R3 according to (ii) are identical or different and each are preferably selected from the group consisting of amines, hydrazides, aldehydes, ketone residues, sulfhydryls, pyridyl disulfides, 2-nitrobenzoic acid disulfides, sulfenylthiosulfate residues, vinylsulfone residues, maleimides, aryl halides, alkyl halides, haloacetyl residues, and thioester residues capable of generating reactive sulfhydryls by treatment with hydroxylamine.

33. System according to claim 31, wherein the oligomeric or polymeric carrier molecules are selected from hydrophilic synthetic or hydrophilic natural polymeric derivatives of nucleotides, saccharides, amino acids, vinyl alcohols, vinylpyrrolidinones, acrylic acids, urethanes, phosphazenes, and copolymer preparations of such polymeric derivatives.

34. System according to claim 22, wherein the detector reagent of (c) comprises a complex of molecules each containing at least one detector unit, at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said detector oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said primer oligonucleotide via a covalent linkage, and at least one fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2, wherein reactive residues R2 and R4 of different molecules are covalently attached to each other.

35. System according to claim 22, wherein the detector reagent of (c) comprises a complex of two different types (I and II) of molecules, type I containing at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said detector oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said detector oligonucleotide via a covalent linkage, and type II containing at least one detector unit and at least two fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2 contained in type I molecules.

36. System according to claim 35, wherein type I molecules contain an enzymatic detector unit and type 2 molecules contain a liposomal detector unit.

37. System according to claim 22, wherein the detector reagents are complexes of detector molecules each containing at least one detector unit and being crosslinked to each other by homobifunctional bridging agents via reactive residues R2 on the said detector molecules and the functional groups on the bridging agents.

38. System according to claim 37, wherein the detector reagents contain enzymatic detector units and/or liposomal detector units.

39. Method for the detection or quantification of a nucleic acid in a liquid sample, comprising the following steps:

(a) providing a solid support having immobilized thereon capture molecules capable of binding to a first part but not to a second part of the nucleic acid to be detected or quantificated or amplicons thereof,

(b) contacting said capture molecules with the liquid sample,

(c) adding detector oligonucleotides having a sequence capable of binding to the said second part of the nucleic acid to be detected or quantified and containing at least one of a first reactive residue R1,

(d) removing unbound reactive detector oligonucleotides,

(e) adding a detector reagent, comprising per molecule at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive R1 residues of the said detector oligonucleotides or to third reactive residues R3 of molecules capable of being bound to the said first reactive residue R1 of the said detector oligonucleotides via a covalent linkage.

(f) removing unbound detector reagent, and

(g) detecting or quantifying the presence of reporter molecules provided or generated by the said detector units.

40. Method according to claim 39, wherein the detector reagent used according to (e) is selected from the group consisting of (i) complexes of molecules each containing at least one detector unit, at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said detector oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said detector oligonucleotides via a covalent linkage, and at least one fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2, wherein reactive residues R2 and R4 of different molecules are covalently attached to each other, (ii) polymeric carrier molecules to each of which more than one detector unit is attached, the detector unit preferably being selected from enzymes immobilized to the polymeric carrier molecules and capable of generating or mediating the generation of colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemically detectable molecules, and (iii) complexes of two different types (I and II) of molecules, type I containing at least one detector unit and at least one second reactive residue R2 capable of forming a covalent linkage to the said first reactive residues R1 of the said detector oligonucleotides or to a third reactive residue R3 of a molecule capable of being bound to said first reactive residue R1 of the said detector oligonucleotides via a covalent linkage, and type II containing at least one detector unit and at least two fourth reactive residue R4 capable of forming a covalent linkage with one of the said second reactive residues R2 contained in type I molecules.

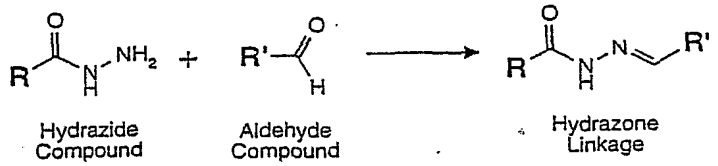
41. Method according to claim 39, wherein the detector oligonucleotides added according to step (c) contain a covalently attached oligomeric or polymeric carrier molecule with multiple reactive residues R1 capable of covalently binding more than one detector reagent.

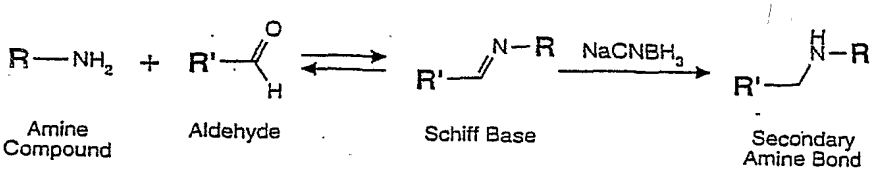
43. Method according to claim 40, additionally comprising

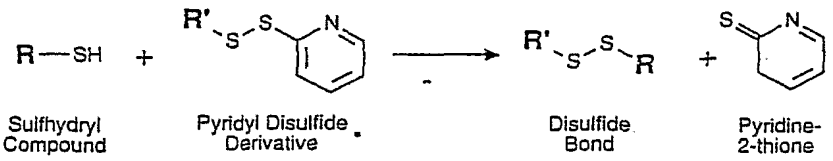
(b') adding polymeric carrier molecules containing (i) at least one reactive residue R3 capable of forming a covalent likage to the said first reactive residues R1 of the said detector oligonucleotides of (c) and (ii) more than one reactive residue R3 capable of forming a covalent linkage to the said second reactive residues R2 of the said detector reagents

after the liquid sample containing the nucleic acid to be detected has been contacted with the said capture molecules and before the said detector reagent has been added.

* * *


Hydrazide Compound
Aldehyde Compound
Hydrazone Linkage


NaCNBH3
Amine Compound
Aldehyde
Schiff Base
Secondary Amine Bond


Sulfhydryl Compound
Pyridyl Disulfide Derivative
Disulfide Bond
Pyridine-2-thione


Sulfhydryl Compound
Iodoacetyl Derivative
Thioether Bond
HI

Fig.1

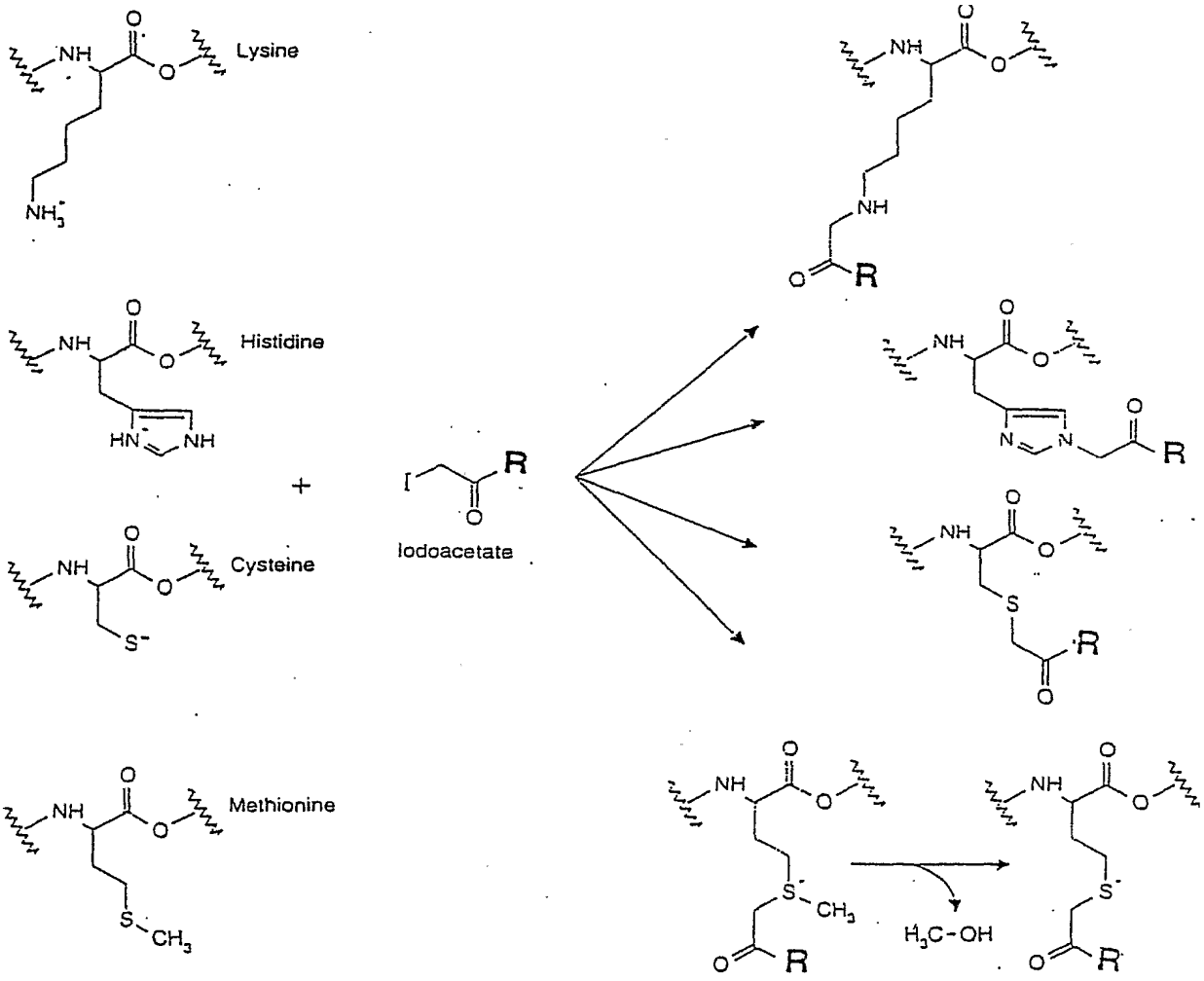

Lysine
Histidine
Cysteine
Methionine
Iodoacetate
R
H_3C-OH

Fig.2

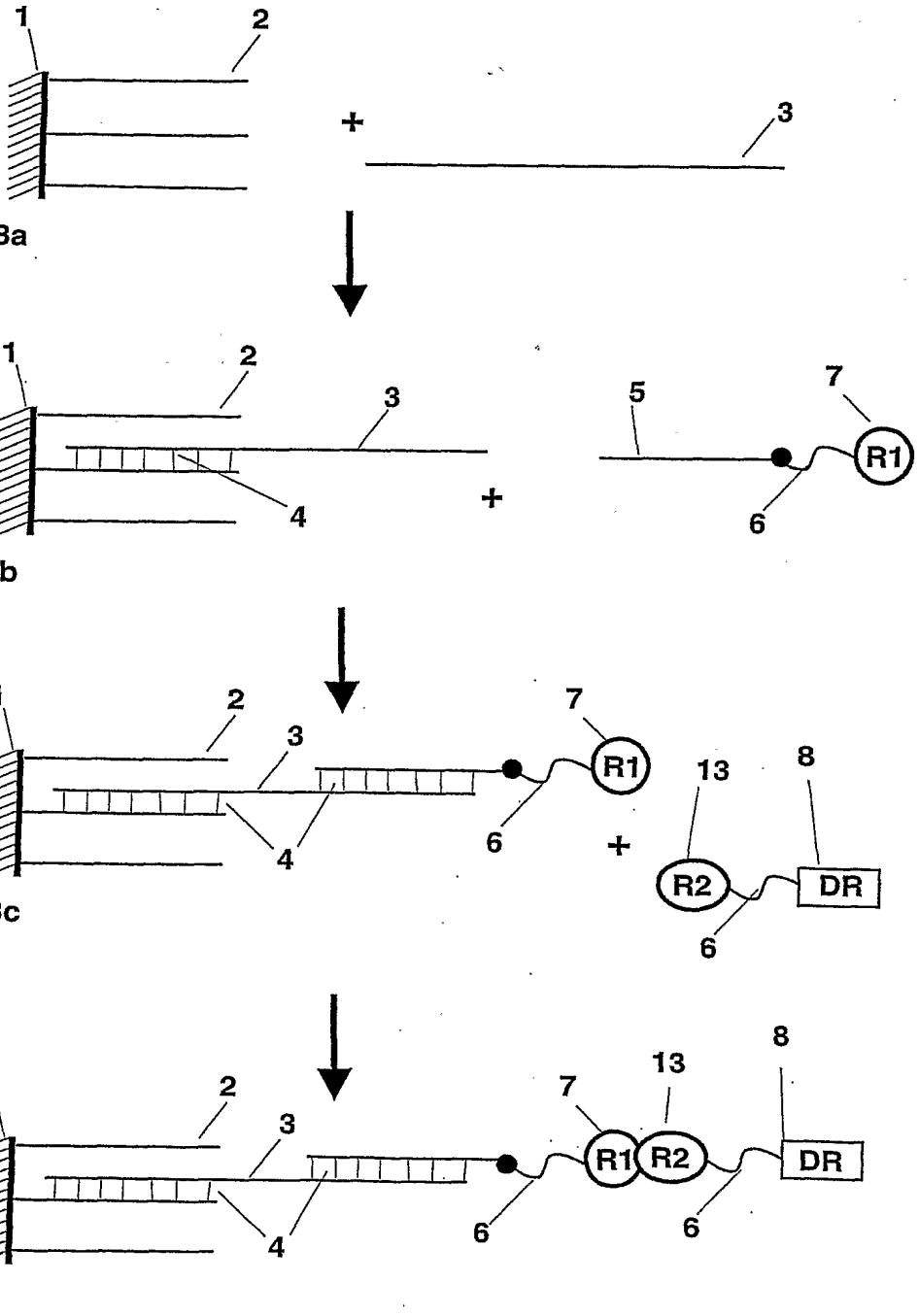

1
2
3
+
Fig.3a
1
2
3
4
+
5
7
R1
6
Fig.3b
1
2
3
4
7
R1
6
+
13
8
R2
DR
6
Fig.3c
1
2
3
4
8
13
7
R1
R2
DR
6
6
Fig.3d

Fig.3

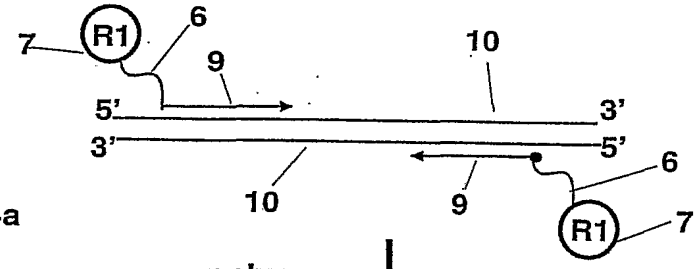


Fig.4a

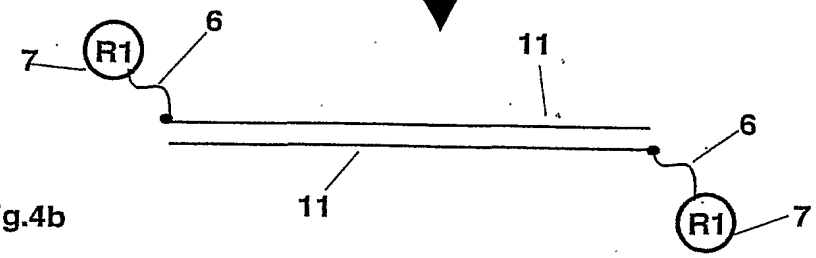


Fig.4b

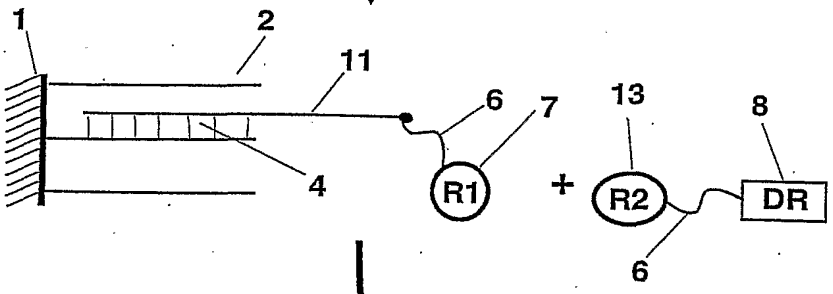


Fig.4c

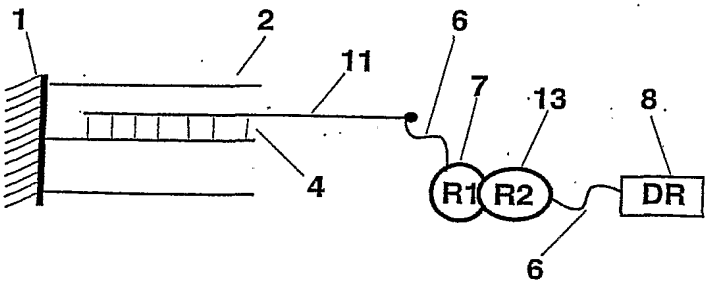


Fig.4d

Fig.4

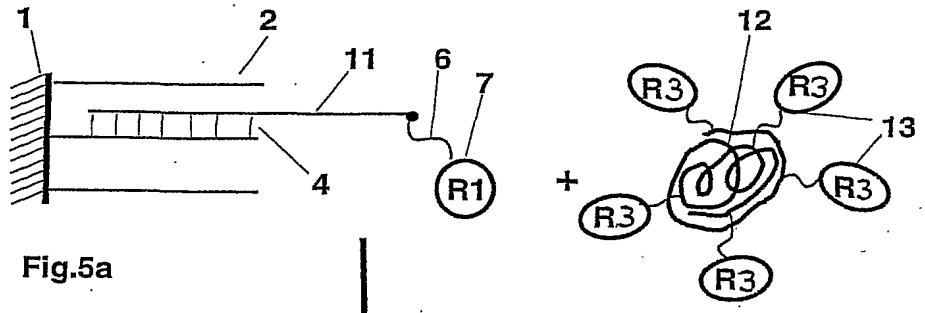


Fig.5a

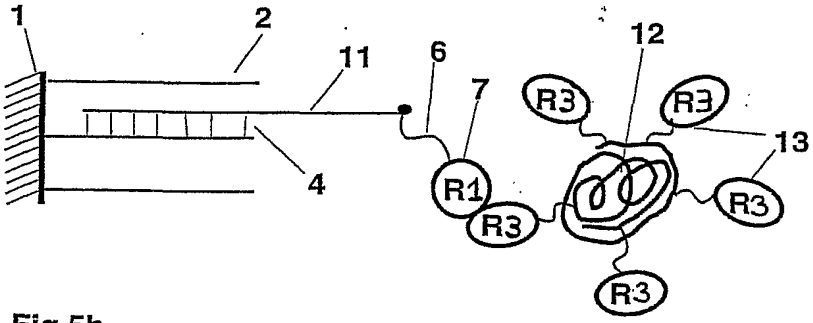
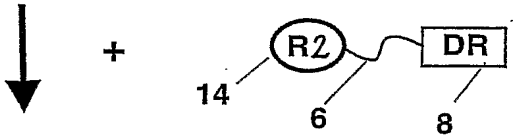


Fig.5b



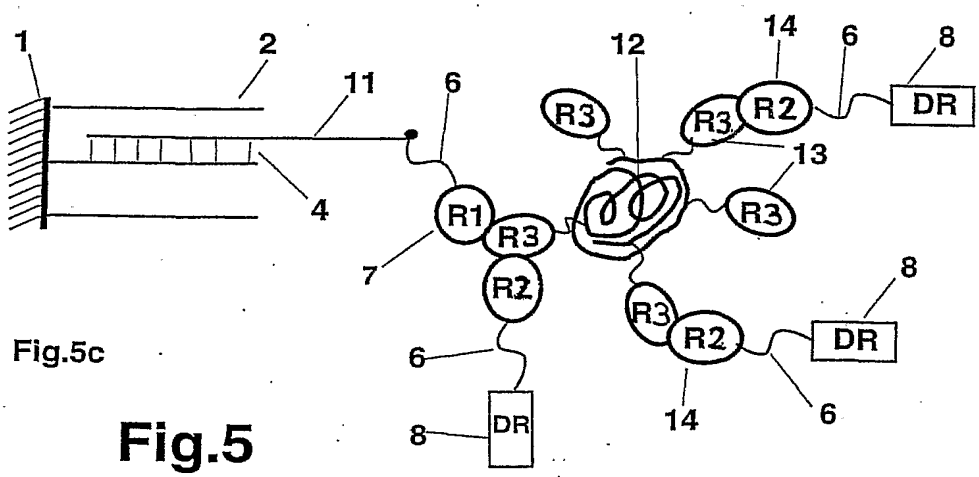


Fig.5c

Fig.5